A RAPID PLATE METHOD OF COUNTING SPORES OF CLOSTRIDIUM BOTULINUM

A. A. ANDERSEN

Western Regional Research Laboratory,[1] *Albany, California*

Received for publication June 4, 1951

Making accurate counts of the spores of *Clostridium botulinum* has been a formidable problem in food bacteriology. A literature survey revealed no standard or widely accepted method. In general, long incubation times have been employed in an effort to count these spores, many of which may remain dormant for weeks or even years.

The present paper describes a fast plate method for counting spores of *C. botulinum*. The method is based on the development of an adequate medium which includes HCO_3^- and the use of a new plating technique which produces anaerobiosis and permits the plate to be counted or examined at any time.

METHOD

Medium (VII). Pork infusion, 800 ml; pea infusion, 200 ml; peptone, 5 g; tryptone, 1.6 g; K_2HPO_4, 1.25 g; soluble starch, 1 g; Na thioglycolate, 0.5 g; N NaOH to pH 7.2; agar, 16 g.

The materials are autoclaved 5 minutes at 121 C and clarified while hot by adding 25 g of "celite"[2] and filtering through Whatman no. 4[2] filter paper with suction. The medium is tubed in 9 and 14 ml quantities and can be stored in a closed container in a freezer. As needed the tubes are autoclaved at 15 pounds for 12 minutes.

The pork infusion is made as follows: fresh, lean pork is ground, stirred into hot distilled water (1 lb per L), and steamed 1 hour. The meat is filtered out on 4 layers of cheesecloth, and the solidified fat is removed after cooling.

The pea infusion is made as follows: fresh or good-quality frozen peas are blended with distilled water (1:1) and steamed 1 hour. The solids are removed by centrifugation and filtration with celite. If not used immediately the pork or pea infusions can be stored at 0 F in enameled cans.

The complete medium also contains $NaHCO_3$ which gives best results if added to the dish at the time of pouring. A freshly prepared solution of 5 g of $NaHCO_3$ per 100 ml is sterilized by pressure Seitz filtration and, if not used immediately, is kept in sterile screw-cap tubes in a refrigerator.

Plating. The anaerobic petri dish used in this work is set up as shown in figure 1. To the petri dish (no. 3162, Corning Glass Works[2]) 0.4 ml of the $NaHCO_3$ solution, 14 ml of the medium, and the inoculum are added and mixed. After

[1] Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

[2] Mention of this product does not imply that it is endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.

solidification of the poured plate, 9 ml of medium are carefully added and a sterile glass plate[3] placed in it to exclude air. Above the glass plate 30 ml of plain agar containing 0.1 per cent Na thioglycolate are added. The dishes are incubated at 30 C until maximum number of colonies develop (usually 40 hours or less). It is recommended that the count be made with an automatic counting pen under a colony microscope (9 ×).

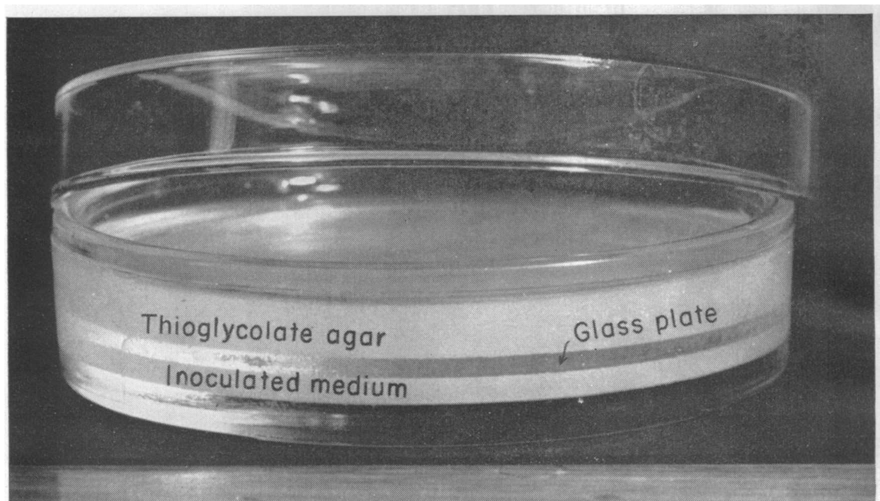

Figure 1. Anaerobic petri dish

EXPERIMENTAL RESULTS

The spores used in developing this method were prepared by growing *C. botulinum* (N.C.A. strain no. 62A) in a pea medium at 30 C for 10 days. The spores were centrifuged out, mixed with soluble starch, dried, and stored *in vacuo* several months preceding this work. A stock suspension, made by blending 0.2 g of the preparation in 100 ml sterile distilled water, was kept in a refrigerator. No reduction in number of viable spores was observed during the few weeks in which the method was developed. After several months, however, a reduction was noted and colony development was somewhat delayed. For each experiment a few ml of the stock suspension were heated 1 to 2 minutes in boiling water and diluted 1:100. Usually 1 ml of this dilution served as inoculum for each dish. Several replicate counts, made by the method as developed, agreed closely and indicated that this amount of inoculum contained approximately 1,200 spores. In the first experiments the counts were in the desired range, but as the technique was improved, higher counts were obtained. Despite these high counts,

[3] Flat plates cut from window glass were used at first, but later it was found that plates in which the outer 2 cm were molded upward 1 to 2 mm were more satisfactory. This facilitated removal of air, and closer fitting plates could be used. The plates were molded by heating them slowly to the softening point between appropriately shaped steel molds. The petri bottom mentioned above is necessary because others are not sufficiently uniform to ensure a good fit with the glass plates.

it was thought desirable for comparative purposes to keep the amount of inoculum constant throughout most of the experiments.

Because of the special treatment given these spores they may be considered different from the suspensions usually used; however, the previously dried spores were thought to be more representative of those encountered in nature. After development, the method also was tested on 6 cultures of *Clostridium botulinum* (62A, 109A, 457A, 115B, 169B, 1267B) and on 3 calibrated spore suspensions (62A, 213B, and PA 3679) received from other laboratories.[4]

Spray dishes, oval tubes, and the anaerobic petri dishes (APD), described under method and shown in figure 1, were used in the development of the technique. Spray dishes were made anaerobic with 12 ml 3 N NaOH or 12 ml 20 per cent Na_2CO_3 and 3 or 4 g of pyrogallol. The oval tubes were made anaerobic by covering the medium with oil or anaerobic agar.

The incubation temperature was 30 C. After the method was developed, it was shown that at this temperature the count was slightly higher than at 35.5 C.

Early in the work the response of *C. botulinum* spores in various media was determined in anaerobic petri dishes. Colony counts on the 1:100 dilution made at 10 days were as follows: BBL anaerobic agar no. 146, 11 − ; pea infusion medium, 22; Difco fluid thiogylcolate + agar, 39; BBL anaerobic agar no. 139, 41; pork-starch medium, 180; Difco fluid thioglycolate (Linden) + agar, 270; Difco brain-heart-infusion, 320; Difco brain-liver-heart, 350; and pork-starch-pea medium, 890. No HCO_3^- was added to any of these media.

From these results it was evident that the pork-starch-pea medium was by far the best, and the pea infusion made an important improvement in the pork-starch medium.

Anaerobic petri dishes and spray dishes were used in the next experiment to determine the effect of adding Na thioglycolate to the pork-starch-pea medium. The spray dishes were made anaerobic with 12 ml of 20 per cent Na_2CO_3 and 3 g of pyrogallol. Addition of Na thioglycolate improved the counts in the anaerobic petri dishes, but a more significant observation was that the spray dishes gave counts several times higher early in the incubation and one and a half times higher even after 12 days. This difference seemed attributable to CO_2 evolved from the Na_2CO_3-pyrogallol solution in the spray dishes.

Another experiment was set up then to determine whether counts in anaerobic petri dishes could be brought up to equal the counts in the spray dishes by the addition of $NaHCO_3$. The *C. botulinum* spore suspension was plated again this time in the pork-starch-pea medium plus 0.05 per cent Na thioglycolate, in both anaerobic petri dishes and spray dishes. To the anaerobic petri dishes various amounts of 10 per cent $NaHCO_3$ solution (sterilized by Seitz filtration) were added. The medium in the spray dishes received no HCO_3^-, but the dishes were made anaerobic with Na_2CO_3 and pyrogallol. In the plates receiving 0.2 ml of the HCO_3^- solution colonies developed with remarkable uniformity and reached a high maximum count within 24 hours. In the HCO_3^--free medium the count

[4] These preparations were kindly furnished by G. M. Dack of the University of Chicago, C. T. Townsend and C. W. Bohrer of the National Canner's Association.

was only 1.5 per cent in 24 hours and 59 per cent in 10 days, compared with the 24 hour HCO_3^- plates. The spray dishes required 3 to 4 days to reach a maximum, which was slightly lower than the HCO_3^- plate count.

The HCO_3^- effect was shown, in another experiment, not to be due to a change in pH. Two series of anaerobic petri dishes were set up, one with various levels of $NaHCO_3$, the other with NaOH sufficient to give corresponding pH values. The addition of 0.1, 0.2, or 0.3 ml of the HCO_3^- solution per plate gave maximum counts in 24 hours (all about equal). In the NaOH series only 1 to 2 per cent of the spores had formed visible colonies in 24 hours and 15 to 40 per cent in 4 days.

TABLE 1

Effect of $NaHCO_3$ on the spore count of Clostridium botulinum

MEDIUM	TYPE OF DISH	COLONY COUNT AT		
		16 hr	24 hr	48 hr
1. Medium VII	APD	0	21	130
2. Medium VII + 0.1 ml 10% $NaHCO_3$	APD	739	1184	1188
3. Medium VII + 0.2 ml 10% $NaHCO_3$	APD	896	1194	1196
4. Medium VII + 0.3 ml 10% $NaHCO_3$	APD	921	1177	1181
5. Medium VII + 0.04 ml N NaOH	APD	0	13	112
6. Medium VII + 0.10 ml N NaOH	APD	0	12	82
7. Medium VII + 0.13 ml N NaOH	APD	0	14	191
8. Medium VII (Na_2CO_3-pyrogallol in dish)	Spray	0	795	1175 (92 hr)
9. Medium VII (NaOH-pyrogallol in dish)	Spray	0	0	0 (42 days)
		18 hr	30 hr	132 hr
10. Medium VII	Oval tube*	0	2	31
11. Same + 0.1 ml 10% $NaHCO_3$	Oval tube*	0	143	172
12. Wynne and Foster medium	APD	0	11	315
13. Same + pea infusion	APD	0	83	616
14. Same + pea inf. + 0.2 ml 10% $NaHCO_3$	APD	905	1197	1195
15. Pea infusion medium (no pork)	APD	0	1	18
16. Same + 0.2 ml 10% $NaHCO_3$	APD	490	932	1043

* Oval tubes received 0.25 ml inoculum; others received 1.0 ml.

In spray dishes it was shown that none of the *C. botulinum*, strain 62A, spores would produce colonies in 42 days if anaerobiosis was produced with pyrogallol-NaOH solution, which absorbs any CO_2. Na_2CO_3-pyrogallol, which evolves CO_2, gave a maximum count in 92 hours.

In a similar experiment with spores of a putrefactive anaerobe, strain no. PA 3679, a maximum count was obtained in 3 days in spray dishes made anaerobic with Na_2CO_3-pyrogallol, but with NaOH plus pyrogallol the count reached only 47 per cent in 21 days.

The HCO_3^- effect was also demonstrated in oval tubes, although this type of apparatus gave considerably lower counts and the counting was much more difficult.

The addition of pea infusion or pea infusion plus $NaHCO_3$ to Wynne and Foster's (1948*a*) medium greatly improved the count. In a medium similar to medium VII (previously described) except that the pork infusion was entirely replaced with pea infusion, the HCO_3^- effect was greatest.

TABLE 2

Effect of sodium bicarbonate on viable spore counts of various cultures of Clostridium botulinum and a putrefactive anaerobe

CULTURE NO. AND SOURCE	MEDIUM	COLONY COUNTS AT			
		20 hr	24 hr	48 hr	168 hr
62A	Medium VII*	216	300	412	421
U. of Chicago	Same + $NaHCO_3$	544	552	551	551
109A	Medium VII	108	450	615	635
U. of Chicago	Same + $NaHCO_3$	651	728	787	788
457A	Medium VII	255	399	412	412
U. of Chicago	Same + $NaHCO_3$	481	498	498	498
115B	Medium VII	575	610	692	689
U. of Chicago	Same + $NaHCO_3$	802	803	803	798
169B	Medium VII	0	45	210	218
U. of Chicago	Same + $NaHCO_3$	447	449	449	449
1267B	Medium VII	0	10	278	286
U. of Chicago	Same + $NaHCO_3$	552	582	610	609
213B	Medium VII	0	48	279	340
N. C. A.	Same + $NaHCO_3$	376	392	416	413
3679	Medium VII	450	514	591	601
N. C. A.	Same + $NaHCO_3$	573	635	653	656
62A	Medium VII	0	21	130	258
N. C. A.	Same + $NaHCO_3$	1096	1194	1196	1196

* Medium VII is listed under Method.

Results of some of the experiments are summarized in table 1.

An experiment was performed to test the effects of starch and of glucose in the presence and absence of HCO_3^- in Medium VII less starch and pea infusion. The results showed that neither starch nor glucose altered the count, which was high when HCO_3^- was present but low when it was absent.

Another experiment indicated that the pH of the medium was not critical when HCO_3^- was added to it. Maximum counts were obtained down to pH 6.0.

Without HCO_3^- the count was low at pH 7.0 and fell sharply as the pH was reduced.

METHOD TESTED

The effect of adding $NaHCO_3$ to medium VII was tested with 8 strains of *C. botulinum* and a putrefactive anaerobe, strain no. PA 3679. These cultures, except for the last one in table 2, were plated directly from the refrigerated cultures after appropriate dilution and heating 1 minute in boiling water. The last two lines (table 2), representing dried spores of *C. botulinum*, strain 62A, are included for comparative purposes.

Table 2 demonstrates the HCO_3^- effect for all of the cultures and shows that the maximum count for each culture is reached within 48 hours in the HCO_3^- medium. Without HCO_3^- the counts lagged far behind and were substantially lower even after 7 days of incubation.

Three spore suspensions, calibrated in other laboratories and by other methods, were counted by the new method. The previous calibrations were: *C. botulinum*, strain no. 62A, 50,000,000; strain no. 213B, 200,000,000; and a putrefactive anaerobe, strain no. PA 3679, 56,000,000. The respective counts by the new method were 225,000,000; 448,000,000; and 126,000,000.

Experiments also showed that colonies developed quickly and uniformly in the HCO_3^- medium whether the spores were several years old or newly grown, whether dried or undried, and whether or not the spores exhibited extreme dormancy in other media. One experiment with spores of strain no. 62A indicated that heat activation of these spores is unnecessary in the HCO_3^- medium. A similar experiment with strain no. PA 3679 indicated that 1 minute of heating in boiling water was beneficial.

DISCUSSION

Although Leifson (1931), Belousskaya (1940), and Wynne and Foster (1948*b*) have reported CO_2 to be beneficial for *C. botulinum*, this information apparently has not been used, or is not being used, in counting methods. This study shows that the addition of $NaHCO_3$ (or CO_2) to the medium is essential for prompt and uniform development of colonies and maximum counts for each of the 8 cultures of *C. botulinum* tested, and for a putrefactive anaerobe. Dried spores appeared to be more dependent on HCO_3^- than those stored in medium. Possibly the CO_2 content of the spores was reduced to a lower level in the dried preparation, and consequently germination could not take place unless it was restored.

Burke (1923) thought dormancy was an inherent property of *C. botulinum* spores. Foster and Wynne (1948) concluded that both dormancy and submaximal counts are due to inhibitory or antigermination substances present in "normal media." The data presented here show that delayed germination and low counts in pork infusion medium are overcome by the addition of HCO_3^- to the medium. The lack of other essential nutrients or presence of inhibitors (in other media) also could cause delay or failure in germination of spores and development of colonies.

Wynne and Foster (1948*a*) reported that they obtained maximum counts in 3 days and eliminated dormancy by the addition of starch to pork infusion medium, although Olsen and Scott (1946) had found that starch increased the count only of severely heat-damaged spores (1 or 2 hours at 100 C; less than 1 per cent remaining viable) but had no effect on unheated spores. We found that starch had no effect on the count of spores heated 1 or 2 minutes in boiling water. It is therefore suggested that the starch assists the spore to overcome heat injury.

Lwoff and Monod (1947) and Ajl and Werkman (1948) found a number of compounds that would replace the CO_2 requirement of *Escherichia coli* and *Aerobacter aerogenes*. Gerhardt and Wilson (1950) found that these compounds would not replace CO_2 for *Brucella abortus*. Wynne and Foster (1948*b*) suggest that complex media contain CO_2 by-passers for *C. botulinum*. The data presented here indicate that if there are CO_2 by-passers, they at least are not present in pork and pea infusions, peptone, and tryptone.

The method has been applied to 9 cultures listed in table 2, and all of them could be counted within 36 hours. This short incubation period, compared with methods employing periods of 21 to 90 days (Schmidt, 1950; Stumbo *et al.*, 1950; Townsend *et al.*, 1938; Williams and Reed, 1942; and Yesair and Cameron, 1942), should greatly facilitate research with *C. botulinum* and putrefactive anaerobes. In addition the higher counts obtained on previously calibrated spore suspensions indicate that the previous methods were lacking in accuracy.

The fact that colony development was rapid and uniform (without stragglers) as long as HCO_3^- was supplied and the fact that variations in other respects did not affect the maximum count suggest that the method may count all viable spores.

SUMMARY

The observation that the addition of $NaHCO_3$ to a pork infusion medium breaks dormancy in spores of *Clostridium botulinum* has led to a new rapid plate method which requires only 24 to 36 hours.

The method involves the use of a petri dish which is made anaerobic with a glass plate and anaerobic agar overlying the inoculated medium. The dish can be conveniently examined at any time.

The method was developed with a spore suspension of *C. botulinum*, strain 62A, and tested with spores of 7 other strains of *C. botulinum* and a putrefactive anaerobe.

The effects of pea infusion, pork infusion, thioglycolate, starch, glucose, and pH on the medium were studied.

The anaerobic petri dish used in this method should also prove useful for work with other anaerobes.

If spray or bray dishes are used in work with *C. botulinum*, it is recommended that Na_2CO_3 rather than NaOH be used with pyrogallol to produce anaerobiosis.

REFERENCES

AJL, S. J., AND WERKMAN, C. H. 1948 Replacement of CO_2 in heterotrophic metabolism. Arch. Biochem., **19**, 483–492.

BELOUSSKAYA, F. M. 1940 Effect of carbon dioxide on growth and toxin formation on *Bacillus botulinus*. Voprosy Pitaniya, **9** (1/2), 61–64 (from English summary).

BURKE, G. S. 1923 Studies on the thermal death time of spores of *Clostridium botulinum*. 3. Dormancy or slow germination of spores under optimum growth conditions. J. Infectious Diseases, **33**, 274–284.

FOSTER, J. W., AND WYNNE, E. S. 1948 The problem of "dormancy" in bacterial spores. J. Bact., **55**, 623–625.

GERHARDT, P., AND WILSON, J. B. 1950 Attempts to replace the added carbon dioxide required by some strains of *Brucella abortus*. J. Bact., **59**, 311–312.

LEIFSON, E. 1931 Bacterial spores. J. Bact., **21**, 331–356.

LWOFF, A., AND MONOD, J. 1947 Essai d'analyse du rôle de l'anhydride carbonique dans lacroissance microbienne. Ann. inst. Pasteur, **73**, 323–347.

OLSEN, A. M., AND SCOTT, W. J. 1946 Influence of starch in media used for the detection of heated bacterial spores. Nature, **157**, 337.

SCHMIDT, C. F. 1950 A method for the determination of the thermal resistance of bacterial spores. J. Bact., **59**, 433–437.

STUMBO, C. R., MURPHY, J. R., AND COCHRAN, J. 1950 Nature of thermal death time curves for P. A. 3679 and *Clostridium botulinum*. Food Technol., **4**, 321–326.

TOWNSEND, C. T., ESTY, J. R., AND BASELT, F. C. 1938 Heat-resistance studies on spores of putrefactive anaerobes in relation to determination of safe processes for canned foods. Food Research, **3**, 323–346.

WILLIAMS, O. B., AND REED, J. M. 1942 The significance of the incubation temperature of recovery cultures in determining spore resistance to heat. J. Infectious Diseases, **71**, 225–227.

WYNNE, E. S., AND FOSTER, J. W. 1948*a* Physiological studies on spore germination with special reference to *Clostridium botulinum*. I. Development of a quantitative method. J. Bact., **55**, 61–69.

WYNNE, E. S., AND FOSTER, J. W. 1948*b* Physiological studies on spore germination with special reference to *Clostridium botulinum*. III. Carbon dioxide and germination, with a note on carbon dioxide and aerobic spores. J. Bact., **55**, 331–339.

YESAIR, J., AND CAMERON, E. J. 1942 Inhibitive effect of curing agents on anaerobic spores. Canner, **94**, 89–92.