The testing of sterilizers

2 Thermophilic spore papers¹

J. C. KELSEY²

From Guy's Hospital Medical School, London

SYNOPSIS Satisfactory spore preparations for testing hospital sterilizers can be made from *B. stearothermophilus* and comparatively simple methods for loading and drying the papers are considered to be valid. Each batch of spore papers should be calibrated by plotting dose-response curves for exposure to steam. A minimum standard in terms of the L.D.50 to moist heat is proposed and confidence limits suggested when such papers are used in pairs or small multiples.

In a previous communication (Kelsey, 1958) attention was drawn to the dangers of testing the efficiency of steam sterilizers by using bacterial preparations of inadequate resistance. It was suggested that when cultural tests are required only preparations of known heat resistance should be used. The present paper describes how such preparations may be made and standardized.

PREPARATION OF SPORE PAPERS

It was decided to use *Bacillus stearothermophilus*, an aerobic spore-bearing thermophile which is well known in the canning industry as the cause of 'flat sours'. Several standard freeze-dried strains are available and the literature suggested that it would have a suitable resistance to heat.

Many techniques were tried in an attempt to produce a good yield of spores. Even using artificial aeration, liquid culture media did not produce such good yields as solid media and eventually the medium chosen was that of Williams, Franklin, Chapman, and Clegg (1957) and called by them 'B.S. medium'. This medium and the details of its use to produce a spore suspension are described below. The technique differs in some respects from that suggested by the Nuffield Provincial Hospitals Trust (1958) and by Farr (1959) but the results have been consistently satisfactory.

The application of 'heat shock' by heating the suspension to 100°C. for five minutes is important. This process applied immediately after harvesting destroys the vegetative bacteria and so enables the viable count to be a true count of spores alone (Curran and Evans, 1947).

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²Present address: Central Public Health Laboratory, Colindale Avenue, N.W.9.

Although the same workers (Evans and Curran, 1960) consider that such spore suspensions once 'heat shocked' retain their viability after prolonged storage at low temperatures (provided the pH is around neutrality), 'heat shock' has always been reapplied just before the impregnation of spore papers, if this is not done at once. This procedure kills any extraneous vegetative organisms that may have contaminated the suspension during storage. It also means that the pre-impregnation viable count of spores will be maximal.

STRAINS The following strains of *Bacillus stearothermophilus* were found satisfactory: N.C.I.B. 8919, N.C.T.C. 10003, N.I.R.D. 202.

INOCULUM Initially a suspension containing spores was made by growing the organism on nutrient agar at 56°C. for five days. In all later productions an existing spore paper was used. The primary inoculum is heated to 121°C. for five minutes, and incubated overnight in broth at 56°C. This culture is then used to inoculate several tubes of broth which are incubated at 56°C. for six hours, and after concentration by centrifugation a heavy suspension is obtained from which the main cultures in Roux bottles are inoculated. It is more satisfactory always to go back to an early batch of spore papers for the inoculum, having found that this gives a good yield, rather than to use the last paper of the previous batch to start the next one.

MEDIUM After much experimentation it was found that good results were consistently obtained by using the 'B.S. medium' of Williams *et al.* (1957) which has the following composition: Oxoid tryptone, 3 g., oxide peptone, 6 g., Yeastrel, 3 g., Lab-Lemco, 1.5 g., New Zealand agar, 25 g., aqueous solution containing 0.001 g. per ml. Mn⁺⁺, 1 ml., distilled water, 1,000 ml. The final pH is adjusted to 7.0. This medium was used to make Roux bottles.

INCUBATION Bottles are incubated for five to seven days at 56°C. The bottles are incubated with the agar downwards and sloped so that excess moisture collects at one end. If necessary, a little sterile distilled water is added from time to time to prevent the drying of the agar. It is important to maintain aerobic conditions by using a loose cottonwool plug or metal cap to close the Roux bottles.

HARVESTING If a stained film shows good sporulation (over 80%) the growth is harvested by shaking with sterile distilled water and sterile glass beads. The crude suspension is heated at 100°C. for five minutes, shaken with beads on a mechanical shaker to break up clumps, and filtered through glass wool. It is washed three times and resuspended in sterile distilled water. A wet preparation is then examined to check that the suspension is smooth and free from clumps. If this is satisfactory the suspension is stored at + 4°C. until required. If long storage is expected a drop of a mixture of equal volumes of toluene and carbon tetrachloride is added, or the spores can be suspended in methanol.

PREPARATION OF PAPERS The suspension is heated at 100°C. for five minutes, counted by the method of Miles and Misra and diluted until the required number of spores is contained in 0.033 ml. The suspension is faintly tinted with the edible dye 'apple green' and used to impregnate Whatman's A.A. discs, 6 mm. in diameter. These discs absorb 0.033 ml. of water.

The discs are placed in a petri dish in shallow layers and flooded with the suspension (the dye prevents any disc being missed). Excess liquid is drained off and the disc dried at room temperature and stored at the same temperature until required.

LOADING THE SPORE PAPERS At first strips of filter paper were used to carry the spores, but cutting them proved very laborious and it was more convenient to use ready prepared discs made of thick filter paper. Such discs are available as Whatman's antibiotic assay (A.A.) discs. They are very uniform and careful experiment showed that the 6 mm. discs regularly absorb 0.033 ml. of water from their air-dried state. This greatly facilitates loading them with spores. The discs can be sterilized by hot air or ethylene oxide, but experience has shown this to be unnecessary. Many hundreds of discs which had not been sterilized before impregnation failed to yield any contaminants more resistant than the *B. stearothermophilus* spores used.

If only a few discs are to be made each can be loaded by dropping from a calibrated dropper or by individual dipping in a suspension. With large batches this is too laborious and a technique in which discs in bulk are flooded with suspension is to be preferred. Such a technique, is, however, subject to criticism on at least two grounds. The disc may not take up the suspension uniformly, those moistened first may abstract spores from suspension soaking through them, so that discs moistened later may contain fewer spores. In addition, when such bulked spores are dried those on the outside of the heap will dry last, moisture will soak through them from

those below and again the spore distribution may be affected. Thus the reliability of the method of bulk loading needed to be investigated.

It was originally hoped that spores could be recovered quantitatively from impregnated spore papers, so that the efficiency of various methods of loading could be directly estimated. Despite repeated attempts no such quantitative recovery technique could be devised. This was because spores were caught up in the fibres of the filter paper. This could be seen by microscopic examination of the pulp resulting from shaking, boiling, blending or other disintegrative procedures. Another approach had to be adopted. A spore suspension was counted and a dilution made so that on average 0.5 to 0.75 spores should be present in 0.033 ml. Thus if a group of discs were impregnated with such a suspension and subsequently cultured most, but not all, should yield growth. If the loading method was not uniform the proportion of positive discs should fall, since some would have several spores and some none.

In a typical experiment a row of 50 A.A. discs was arranged in 'rouleaux' formation in a petri dish. A spore suspension was prepared as described above and tinted green with the edible dye 'apple green' which had previously been shown not to be toxic. One end of the row of discs was flooded with suspension and more was added until the dye had spread to the far end. The first, middle, and last 10 discs were cultured in nutrient broth for five days at 56°C.

TABLE I

	Number Yielding Growth
First 10	8
Middle 10	8
Last 10	7
Mean 7.7	$\chi^2 = 0.88$ $P = 0.6$

This difference is not significant.

In another typical experiment 20 A.A. discs were individually dipped in the suspension and 20 were impregnated in bulk by adding suspension to them in a universal container until the dye appeared to have reached all the discs and a little surplus fluid was visible. Each group was cultured as before.

TABLE II

	Number Yielding Growth
Individual dipping	18
Bulk flooding	14
Mean = 16	$\chi^2 = 1$ $P = 0.3$

This difference is not significant.

It was concluded from these and similar experiments that the bulk impregnation of spore papers is a valid procedure.

DRYING THE SPORE PAPERS Apart from the criticism mentioned previously, that discs flooded and dried in bulk might have their spore loads altered in the drying process, it is possible that while the impregnated papers are being dried a proportion of the spores will be killed

so that the final spore population may not be that intended. Two experiments were made to test these points. In the first, 40 A.A. discs were impregnated in bulk using the same suspension as previously and dried overnight at room temperature. Twenty were arranged in a heap; 20 were arranged individually in rows. All were cultured for five days at 56°C.

TABLE III

Number Yielding Growth

Heap	20
Rows	14
Mean = 17	$\chi^2 = 1.09$ $P = 0.3$

This difference is not significant.

In the second experiment a batch of discs was impregnated in bulk and groups of 20 were cultured wet and after drying overnight at various temperatures.

TABLE IV

Number Yielding Growth

Cultured wet	18
Dried at room temperature	16
Dried at 37°C.	13
Dried at 56°C.	14
Mean = 15	$\chi^2 = 1$ $P = 0.8$

This difference is not significant.

Thus the use of dried discs would seem to be justified.

STORAGE OF SPORE PAPERS Spore papers made in November 1957 were still viable in June 1960; it was not possible to compare precisely their heat resistance at the beginning and end of this period, since the need for relatively precise standardization methods was not appreciated until late in their history, and at first an 'all-or-none' method of standardization was used. It is probable that on this standard there is a slow fall in resistance, but, as discussed below only further work using more refined techniques will show whether this is true.

RECOVERY OF SPORE PAPERS In practice spore papers are inserted into test loads and cultured after exposure to a sterilization procedure. It was thought possible that the method of culture was important. Experiments were therefore made to test various media, temperatures of incubation, and conditions of culture, using a group of discs impregnated with a nominal load of 100,000 spores and heated for a time calculated to yield a proportion of positive cultures just below 100%. A representative set of results is given below.

TABLE V

Results (Groups of 20 Discs) Number Yielding Growth

Nutrient broth $\frac{1}{2}$ in. depth 50°C.	20
Nutrient broth $\frac{1}{2}$ in. depth 56°C.	20
Nutrient broth $\frac{1}{2}$ in. depth 60°C.	20
Nutrient broth $\frac{3}{4}$ in. depth 56°C.	19
Oxoid dextrose tryptone broth $\frac{1}{2}$ in. depth 56°C.	20
Nutrient broth 56°C. immediately	20
Nutrient broth 56°C. after 1 week	17
Nutrient broth 56°C. after 2 weeks	20
Nutrient broth 56°C. after 3 weeks	18
Nutrient broth 56°C. after 4 weeks	19

It will be seen that the cultural conditions used did not affect the results and that there is no evidence that discs should be cultured within a day or so of exposure.

In all experiments made with spore papers incubation was continued for five days, although it was only very rarely that new positive cultures appeared later than the third day. It is possible that had incubation been extended an occasional new positive would have appeared, but five days was chosen as a reasonable time in practice. It was thought that it was more important to calibrate the overall method of using cultural tests than to try to establish the limits of late recovery, which could easily become quite unrealistic.

STANDARDIZATION OF SPORE PAPERS

Since the object of using thermophilic spore preparations was to ensure adequate heat resistance, it was necessary to lay down some standard for such preparations. This problem became the more pressing when spore papers were commercially produced.

The only precedent available was the standardization of spore suspensions for use in the canning industry. Many elaborate and precise methods are available and a suspension can be characterized by its survival curve, by its thermal resistance curve, or by various mathematical indices (*e.g.*, Schmidt, 1957). All of these suffer from the same disadvantage: they apply to a smooth suspension rather than to the impregnated spore paper. If it is assumed that the resistances of individual spores are normally distributed, the overall resistance of a very large sample should be relatively uniform, since at least one very resistant spore should be in the total spore population comprising the sample. In terms of the making of spore papers this would mean smooth suspensions and the uniform loading of papers. In fact suspensions are seldom smooth and in the previous section it was seen that the processes of loading and drying the paper discs themselves are possible sources of irregularity.

Had it been possible to recover spores quantitatively from the spore papers after impregnation, survival curves, relating the numbers of surviving spores to the time of exposure at a given temperature, could have been plotted and standardization could have been defined in terms of the decimal reduction time.

Because of these difficulties it was decided to treat each spore paper as a biological unit and to attempt to plot dose-response curves against exposure to heat. From this a total picture of the behaviour of the batch of spore papers could be obtained.

It was thought that from this information an L.D. 50 could be deduced which could provide a standard for heat resistance.

In these experiments groups of spore papers, individually wrapped in paper envelopes, were exposed to steam at 121°C. for various times in a high-speed instrument sterilizer, which conformed in all significant details to British Standard Specification 3233:1960.

GENERAL TRIAL OF THE METHOD Determinations were made on the same batch of spore papers on three separate occasions, with the following results:

TABLE VI

Number in Group	5 May 60		11 May 60		12 June 60	
	20	%	10	%	20	%
Survivors 2 min.	20	100	10	100	20	100
Survivors 4 min.	16	80	9	90	20	100
Survivors 6 min.	7	35	4	40	7	35
Survivors 8 min.	0	0	0	0	0	0

Dose-response curves plotted from these results are shown in Figure 1. There is a close correspondence between the curves. The L.D. 50 is virtually the same for each, namely, about 5½ minutes.

COMPARISON OF SEVERAL BATCHES OF SPORE PAPERS Since it seemed that the dose-response curve was likely to give useful results, three commercial suppliers of spore papers were asked for 100 papers each. Dose-response curves at 121°C. were then plotted, using groups of 20 papers at each exposure, for the three commercial batches, P, Q, and R, a current batch 4, a very recently prepared batch 5,

FIG. 1. Dose-response curve of batch 4 spore papers determined on three separate occasions.

and the oldest surviving batch A3, which was made in November 1957 and was 30 months old at the time of retesting. The results are given in the table below.

TABLE VII

Strain	Date	Origin
A3	Nov. 57	J.C.K.
4	Nov. 59	J.C.K.
5	July 60	J.C.K.
P	May 60	Commercial
Q	April 60	Commercial
R	May 60	Commercial

TABLE VIII

Survivors out of 20							
Min.	A3	4	5	P	Q	R	
2	16 (80)	20 (100)	20 (100)	20 (100)	20 (100)	20 (100)	
4	11 (55)	20 (100)	19 (95)	9 (45)	20 (100)	13 (65)	
6	4 (20)	7 (35)	10 (50)	1 (5)	19 (95)	0 (0)	
8	0 (0)	0 (0)	4 (20)	0 (0)	6 (30)	0 (0)	
10	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	

Percentages are in brackets

It will be seen that tolerably smooth curves are given by each batch, considering the necessarily rather crude technique used. The top of the curve for A3 is incomplete; exposures of less than two minutes were not considered reliable, and were not attempted.

The curves for batches P, Q, and 4 were roughly parallel. Each of these batches was made from the same strain of *B. stearothermophilus*, N.C.I.B. 8919. Batch R, made from N.C.T.C. 10003, and Batch 5, made from N.I.R.D. 202, do not show a very different slope, but Batch A3 made from N.C.T.C. 9996 shows a much more gentle slope than the others.

Two possible explanations for these differences could be given. The curve might be characteristic of the strain used, or of the age of the spore papers. A3 being two years older than the other batches. There is not enough evidence to settle this point finally, but the first hypothesis seems preferable. First N.C.T.C. 9996 was discarded very early in the spore making programme just because it was found to give very variable results. Secondly, although standardization of papers was not attempted until 1960, there is a record of survival experiments made when the papers were new. These were crudely performed, only six papers being used per group and the heating was done in an unmodified pressure cooker. At 121°C. six out of six papers survived five minutes and three out of six survived 10 minutes.

When these are plotted, to give the continuous line on Fig. 2, the slope does not differ widely from that obtained two years later. Only repeated detailed calibration of large batches of papers over several years will settle this point.

FIG. 2. Dose-response curves of different batches of spore papers: tested June and July 1960. (Also plotted is the rough curve obtained from two isolated observations on batch A3 in December 1957.)

USE OF THE L.D.50 TO DEFINE A STANDARD At present it seems reasonable to assume that the slope of the dose-response curve is characteristic of the strain and remains reasonably constant at least for a period of months. If this is accepted the L.D.50 could be used to characterize a batch of spore papers and a minimum L.D.50 could be required from a batch made from that strain. The problem is then to choose such a minimum standard. Before this can be attempted the degree of safety expected from a particular value of L.D.50 must be considered.

In Fig. 1 the L.D.50 for the curve of 11 May 1960 is five and a half minutes. At four minutes 90% survival was obtained and at seven minutes 10%. It could thus be claimed that if a single spore paper were to be used in a test sterilization at 121°C. and if it was in fact sterilized, there is a probability of 0.9 that the load had received at least four minutes' exposure at 121°C. Expressed differently, one such test in 10 might give a falsely reassuring result if four minutes is taken as an adequate time. If a pair of papers were to be used and both were in fact sterilized, a false result could be given once in 100 tests. For three papers the chance would be once in 1,000 tests and so on.

From the same curve it might be deduced that at the three minutes' level the probability is 0.98, but the accuracy of deductions from such sigmoid curves is least near the ends and it is probably unwise to go above 90% or below 10%.

POSSIBLE USE OF PROBABILITY CURVES More refined statistical methods would allow more accurate probabilities to be deduced.

The analysis by the method of Schmidt (1957) following Reed (1936) of the pooled results on Batch 4 spore papers is as follows:

	5 May	11 May	12 June	Total	
Number in Group	20	10	20	50	%
Survivors 2 min.	20	10	20	50	100
Survivors 4 min.	16	9	20	45	90
Survivors 6 min.	7	4	7	18	36
Survivors 8 min.	0	0	0	0	0

Time	+	-	m	n	p
2	50	0	113	0	0.9
4	45	5	63	5	8.6
6	18	32	18	37	67
8	0	50	0	87	99

where + = number growing

- = number sterile

m = running total of + from bottom

n = running total of - from top

and $p = \text{probability of sterility} = \frac{n+1}{m+n+2}$

These are shown plotted on probability paper in Fig. 3. The points are not linear but the best straight line has been drawn. It will be seen that the L.D.50 is as before, five and a half minutes.

The probability of survival at four minutes is 0.93 and at three minutes 0.98, and at two and a half minutes 0.99. From this it could be stated that if

FIG. 3. Probability of sterility or growth plotted against time of exposure at 121°C. Pooled results on batch 4.

such a paper was sterilized, only on one occasion in 100 would it have received less than two and a half minutes. For a pair of papers it would be once in 10,000 times and so on.

The plot of Fig. 3 is from pooled data, and represents groups of 50 papers heated for each time interval. Similar plots from the commercial batches, limited to 20 papers per group, were too irregular to use. The more refined analysis is sensitive to smaller variations and needs larger groups of test object than are likely to be available in practice: it also involves much labour. It is probably better to continue to use the cruder but more convenient dose-response curve to define a batch of spore papers.

A SIMPLE STANDARD PROPOSED From the data presented in the earlier paper (Kelsey, 1958), three minutes at 121°C. might be considered as a bottom limit for a test preparation. It would be preferable to take five minutes as giving a wider safety margin. Figure 4 shows the pooled dose-response curve of the three estimations of Batch 4. This gives a 0.9 probability at four minutes. Parallel to this curve are drawn hypothetical curves (W and X) to give the same 0.9 probability at three minutes and five minutes. From these L.D.50s of four and a half and six and a half minutes are deduced, which will be the minimum to give a 0.9 probability of surviving three and five minutes, respectively.

Thus, for this strain a satisfactory batch of spore papers should have a L.D.50 of at least six and a half minutes at 121°C. (or four and a half minutes if the

less strict standard is accepted). Such a batch, if used in pairs, will give a potentially misleading result once in 100 times.

In view of the general crudity of the methods used to prepare and test spore papers two further simplifications can probably be accepted. First, a rounded figure of five minutes for the minimum L.D.50 could be used. Secondly, the standard could be defined more simply by requiring that after being heated for five minutes at 121°C. not less than 50% of a group of, say, 25 papers, should survive, always assuming that the dose-response curve did not depart significantly from that of the strain just considered, namely, N.C.I.B. 8919. Such papers used in pairs would give an assurance of an exposure to 121°C for a minimum of about three and a half minutes with a 1 in 100 chance of being misleading. This can be deduced from the third hypothetical curve Y. Since a sterilizer is unlikely to be judged on a single test, serious error is unlikely to occur.

INFLUENCE OF THE SPORE LOAD PER PAPER UPON L.D.50 If this crude but simple standard of a minimum survival of 50% after five minutes at 121°C is accepted, makers of spore papers will need to consider how this standard can be reached. Intuitively it seemed that one way to alter this resistance would be to alter the number of spores per paper, which up to now had been arbitrarily standardized at 100,000.

A further experiment was made in which a single spore suspension was used to load papers with a nominal 1,000, 10,000, and 1,000,000 spores respectively. The resulting papers were then heated at 121°C. in steam for various times.

FIG. 4. Dose-response curve from pooled results on batch 4 with hypothetical curves to show three possible standards.

		1,000		100,000		1,000,000	
		20	%	20	%	20	%
Survivors	2 min.	12	60	20	100	20	100
Survivors	4 min.	4	20	18	90	20	100
Survivors	6 min.	2	10	8	40	18	90
Survivors	8 min.	0	0	0	0	7	35
Survivors	10 min.	0	0	0	0	0	0

These results are plotted in Fig. 5.

The dose-response curves are approximately parallel. At 1,000 and 1,000,000 spores per paper the L.D.50 is two and a half and seven and a half minutes respectively.

Thus in practical circumstances once a suspension has been made, some of it could be used to make trial spore papers loaded with several different numbers of spores. Batches of these could be tested for survival of five minutes at 121°C. and from the results of this relatively simple experiment a loading

FIG. 5. Dose-response curves for different levels of loading papers with spores.

could be selected which would give the production batch the required minimum survival.

It is suggested that if strains of *B. stearothermophilus* are used whose behaviour does not differ significantly from that of N.C.I.B. 8919, a recently made batch of spore papers having a survival of not less than 50% after exposure to steam at 121°C. for five minutes will give acceptable results, if used in pairs and if the possibility of error once in 100 tests is recognized. Until the ageing of spore papers has been studied in greater detail it is probably advisable to recalibrate each batch every six months.

All experience gained since the publication of the earlier paper has confirmed two of the opinions therein expressed. First, for routine sterilizer testing chemical indicators are the most generally useful: bacteriological tests should be kept for special purposes and should be made by experienced people.

Secondly, where spore preparations are used these must be carefully standardized, and ideally should be provided by a reference laboratory or from commercial sources.

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ADDENDUM

Since the above paper was completed a further calibration of spore papers has been made, five to six months after the first. No further samples of P were available.

Batch No.	Interval (mth.)	Old L.D. 50 (min.)	New L.D. 50 (min.)
A3	6	4½	6
4	5	5½	5½
5	5	6	7
Q	5	6	7
R	5	4½	4½

The slopes of the new calibration curves did not differ markedly from those previously obtained.

These results suggest that spore papers once found satisfactory have a shelf life of at least six months. Further estimations on these papers will be made from time to time.

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