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A standard technique for the isolation of *Salmonella* from animal feeds

By P. J. SMITH
Veterinary Investigation Centre, Crown Street, Liverpool L7 7EH

on behalf of the Salmonella Working Group, Techniques Subcommittee*
of the Ministry of Agriculture, Fisheries and Food

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SUMMARY

Two trials were carried out by 9 and 47 laboratories to evaluate the reproducibility and sensitivity of a standard technique for the isolation of salmonellas from animal feeding stuffs.

The trials involved the examinations of test samples containing known numbers of salmonellas by a buffered peptone water pre-enrichment technique. Statistical analysis of the results of the trials showed that the method was capable of detecting salmonellas in most probable numbers as low as 2·3 per 100 g and that no significant differences in performance were observed between laboratories.

The standard technique described is recommended for the examination of animal feed and conforms to international recommendations.

INTRODUCTION

The introduction of a standard technique for the isolation of *Salmonella* from animal feed is necessary not only to achieve harmonization between laboratories but also for the purpose of national legislation and international agreements. Acceptable bacteriological standards for feed monitoring can be defined only when a reliable isolation technique is available.

Edel & Kampelmacher (1969, 1973, 1974), in evaluating a standard technique for salmonella isolation, demonstrated that there was significant inter-laboratory variation in results. Although the adoption of a standard technique did not bring about any improvement in laboratories with considerable experience in isolating and identifying salmonellas, it led to improved results in less experienced laboratories.

This paper describes trials carried out in Great Britain to introduce a standard technique to laboratories which may be asked to isolate *Salmonella* from animal

* The subcommittee consisted of the following members: P. S. Dawson, MAFF Regional Animal Health Office, Cambridge; J. H. McCoy, Public Health Laboratory, Hull; K. C. Meldrum, MAFF Animal Health Office, Leamington Spa; P. J. Smith, MAFF Veterinary Investigation Centre, Liverpool: W. J. Sojka, MAFF Central Veterinary Laboratory, Weybridge; B. M. Williams, MAFF Veterinary Investigation Centre, Carmarthen; C. Wray, MAFF Central Veterinary Laboratory, Weybridge.

feed and to assess its sensitivity and reproducibility. The study consisted of a pilot trial between 9 laboratories and a later trial between 47 laboratories.

MATERIALS AND METHODS

Pilot trial (9 *laboratories*)

Feed samples

Meat and bone meal and fish meal samples from which *Salmonella* had been isolated on routine monitoring tests were stored at 4 °C until required. For the purpose of the trial the stored positive feed samples were pooled in a large polythene bag and mixed by shaking and rolling for 1 h (sample A1). A similar composite sample was made from feeds which had failed to yield *Salmonella* on culture (sample B1).

Each sample was divided into 150 g lots to provide sufficient material for the trial isolation technique and for the methods used normally by each participating laboratory. One lot each of samples A1 and B1 was sent by post to each of 9 laboratories (A–J) including the organizing laboratory, with instructions to store the samples at 4 °C until the day of testing.

Standard technique

Instructions on the standard technique, together with all the dehydrated media and solutions required for the examination, were sent to each laboratory 2 weeks before despatch of the test samples. The media and reagents used were of the same batches. Specific details upon incubation times and temperatures were given. The importance for operators to gain familarity with the method before the trial began was emphasized. Test samples were posted on a Friday in the expectation that they would arrive at each laboratory the following Monday, thereby reducing the risk of any variation in the bacterial content of samples during transit.

The media used in the pilot study were those detailed in Appendix 1 with the addition of selenite broth (SB) and deoxychocolate citrate lactose sucrose agar (DCLS). Selenite broth base (Oxoid CM395), sodium biselenite (Oxoid L21) and DCLS agar (Oxoid CM393) were reconstituted according to the manufacturers' instructions. Examinations of the samples began on a Monday when 2×25 g of sample were each added to a jar containing buffered peptone water (BPW). After 18 h incubation at 37 °C 100 ml volumes of SB and Muller Kauffmann tetrathionate broth, Oxoid CM393 (MKTB) were inoculated each with 10 ml of the pre-enriched samples and incubated at 43 °C. After 24 and 48 h incubation, the enrichment broths were subcultured to two plates of brilliant green agar (BGA) and single plates of bismuth sulphite agar (BSA) and DCLS. Up to three suspected salmonella colonies were selected from each plate for examination. A portion of each colony was inoculated into triple sugar iron agar (TSI) (Oxoid CM278); into urea broth and on a nutrient agar slope. Urease negative colonies were examined by slide agglutination with polyvalent 'O' and 'H' (phase 1 and 2) salmonella agglutinating sera.

Individual laboratory methods

Laboratories were asked to test the samples by their normal salmonella isolation methods in addition to the standard technique. The methods used by the 9 laboratories showed considerable variation. The quantity of sample examined varied from 10 to 25 g. Some laboratories used pre-enrichment in peptone water or quarter strength Ringer's solution for up to 5 h before addition to double strength SB. Others used direct enrichment in peptone water or SB. Incubation temperatures were either 37 °C or 43 °C. Subcultures were made on single plates of either BGA, BSA or deoxycholate agar.

Second trial (47 *laboratories*)

Feed samples

Known positive and negative bulk samples were prepared as described previously. From each sample 6 lots of 18 g were inoculated into jars containing 150 ml BPW and examined by the standard technique. Most probable number (MPN) values were calculated using the formula

$$\lambda = \frac{-c \log_e (n-r)/n}{w}$$

where λ = MPN/100 g; r = number of jars found to be positive; n = number of jars tested; c = total amount of food in which MPN is to be expressed; w = amount of food in each jar.

The MPN of the bulk positive sample (A2) was found to be 10/100 g, of the bulk negative sample (D2) < 1/100 g. Samples A2 and D2 were mixed in varying porportions to produce samples B2 with estimated MPN 2/100 g and C2 with estimated MPN 3·3/100 g.

Each of the bulk samples A2, B2, C2 and D2 was divided into 150 g lots. From each sample 4 lots selected at random were sent by post to laboratory 26 and one lot to laboratory 20 for MPN estimations. Two weeks later a further 3 lots of each sample were sent to laboratory 26 and one lot to laboratory 20 for MPN estimations. The mean of these 9 MPN counts for each test sample gave the following MPN values per 100 g of sample: A2, 8·4; B2, 2·3; C2, 2·8 and D2, < 1.

Two weeks after preparation one lot of each test sample was despatched to all 47 laboratories for examination by the standard technique with instructions for the recipient laboratory to store the sample at 4 °C until the start of the examination. The 47 laboratories selected for the trial included Government laboratories likely to undertake future feed examination programmes and other laboratories engaged in the examination of feeds over a number of years. It was considered that four samples of varying degrees of contamination would allow an estimate of the sensitivity of the proposed technique to be made.

Table 1. *Laboratory isolations of salmonella using a standard technique – pilot trial*; (*Sample* A1)*

Lab.	Sample	SEL (24) BSA	SEL (24) BGA1	SEL (24) BGA2	SEL (24) DCLS	MKTB (24) BSA	MKTB (24) BGA1	MKTB (24) BGA2	MKTB (24) DCLS	SEL (48) BSA	SEL (48) BGA1	SEL (48) BGA2	SEL (48) DCLS	MKTB (48) BSA	MKTB (48) BGA1	MKTB (48) BGA2	MKTB (48) DCLS	Serotypes isolated	Own method
A	1	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	Group B	—
	2	–	–	–	–	+	–	–	–	–	–	–	–	+	–	–	–		
B	1	–	+	+	–	+	+	+	+	–	+	+	–	+	+	+	+	Group B, E	—
	2	Results entered as similar																	
C	1	–	–	–	–	–	–	–	–	+	–	+	+	+	+	–	+	Group B	—
	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–		
D	1	+	+	+	–	–	+	+	–	–	–	–	+	+	–	–	–	*S. bredeney*	+
	2	Only one tube inoculated																	
E	1	–	–	–	–	–	+	+	–	–	–	+	–	–	+	+	+	*S. bredeney*	+
	2	–	+	+	–	+	–	–	–	+	+	+	–	+	–	–	+	*S. isangi*	
F	1	–	–	+	–	–	+	+	–	–	–	+	–	–	+	+	–	Group B	—
	2	Only one tube inoculated																	
G	1	+	+	+	–	+	–	–	–	+	+	+	–	–	–	–	–	*S. bredeney*	Not done
	2	+	–	+	–	–	–	–	–	+	–	+	–	–	–	–	–		
H	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	—	Not done
	2	Only one tube inoculated																	Not done
J		Counts of 4 MPN/100 g *S. bredeney* and *S. give*																*S. bredeney* *S. give*	

* Sample B1: all laboratories recorded negative results.

Methods

The standard technique recommended is detailed in Appendix I. In view of the results of the pilot study, the media used were reduced to BPW, MKTB, BGA and BSA. The methods of identification of suspicious colonies were left to the choice of individual laboratories but guidelines were given.

RESULTS

Pilot trial

Comments received from the participating laboratories in the pilot trial showed that experience gained before the trial in the use of media was of major importance in the isolation and identification of salmonellas and that unfamiliarity with the standard technique may have affected some of the results. Time was needed to become familiar with the technique, and in particular with the colonial appearance of different organisms on the plating media. Generally laboratories had difficulty in interpreting the reactions on TSI and success was once again dependent upon the experience of the user.

Table 1 shows the results obtained by the 9 laboratories in the pilot trial. Laboratory J, by a multiple tube technique using tetrathionate broth B (Rolfe, 1946) and bismuth sulphite agar, reported an MPN of salmonellas of 4 per 100 g in the sample received, but did not examine the sample by the standard technique. Three laboratories examined only one 25 g sample. Two laboratories did not examine the sample by their normal method. Four laboratories failed to isolate salmonellas from the sample by their normal method but only one laboratory failed to isolate salmonellas from the sample using the standard technique.

There was considerable variation between laboratories in the frequency with which salmonellas were detected on the different media. It was clear, however, that the combination of enrichment in SB with subculture to DCLS was the least satisfactory, and also that subcultures from MKTB to DCLS were effective only after 48 h enrichment. BGA appeared to be the most satisfactory of the three media used for subculture.

Second trial

Table 2 shows the results of examination of two 25 g lots from each sample by the laboratories taking part in the trial. Only one laboratory failed to isolate salmonellas from sample A2, the positive control sample. The negative control sample D2 was found positive by 5 laboratories. This was not unexpected as the sample was regarded as negative on the basis of only nine examinations by the MPN method. Salmonellas were found in sample B2 by 23 laboratories and in sample C2 by 31 laboratories.

A comparison of expected total negative tubes for each sample in the second trial calculated from the formula $(n-r)/n = (-w/e)^e$ was made against the observed number of negative tubes for all laboratories (Table 3). The total number of tubes examined for each sample was 94. Considering the low numbers in the samples it was not expected that all laboratories would isolate salmonellas

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Table 2. *Laboratory isolations of salmonella using a standard technique – second trial*

Number of tubes* found positive for each sampls.

	Sample			
Lab code	A2	B2	C2	D2
01	2	1	0	0
02	2	0	0	0
03	0	0	2	0
04	2	2	2	0
05	1	1	0	2
06	2	1	0	0
07	1	0	1	0
08	2	1	1	0
09	2	2	0	0
10	2	0	1	0
11	2	2	0	0
12	2	2	1	0
13	1	1	2	1
14	2	2	2	1
15	2	2	1	0
16	2	0	2	0
17	2	0	0	0
18	1	1	2	0
19	2	1	0	0
20	2	0	2	0
21	2	0	1	0
22	2	1	2	2
23	2	0	0	0
24	1	2	1	0
25	1	0	2	0
26	1	0	1	0
27	1	2	1	0
28	2	2	2	1
29	1	0	2	0
30	2	0	2	0
31	2	0	0	0
32	2	0	0	0
33	2	0	0	0
34	2	0	0	0
35	2	1	2	0
36	1	1	1	0
37	2	0	0	0
38	2	0	1	0
39	2	0	1	0
40	2	0	2	0
41	2	0	2	0
42	2	2	2	0
43	1	0	1	0
44	1	0	0	0
45	2	1	1	0
46	2	2	2	0
47	2	2	0	0

* Each laboratory examined two 25 g amounts of each sample.

Sample	Mean MPN
A2, Positive control	10/100 g
B2, Test sample	2/100 g
C2, Test sample	3·3/100 g
D2, Negative control	< 1/100 g

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Table 3. *Comparison of estimated and observed results by means of MPN values – second trial*

	Sample			
	A2	B2	C2	D2
Calculated mean MPN/100 g	10	2	3·3	0
Mean MPN/100 g feed estimates from 9 counts	8·4	2·3	2·8	< 1
Mean MPN/100 g estimated from results of trial	7·8	1·8	2·8	0·3
Expected no. of negative samples*	12	53	47	> 73
Observed no. of negative samples*	14	59	46	87

* Total samples examined = 94.

Table 4. *Comparison of BGA and BSA agar in second trial (24 and 48 h readings combined)*

Plate				Sample			
BGA		BSA		A	B	C	D
1	2						
+	+	+		71	32	35	3
+	+	−		1	0	6	1
+	−	+		3	1	2	1
−	+	+		1	0	3	0
+	−	−		2	1	1	1
−	−	+		2	1	1	1
−	−	−		14	59	46	87
			Total	94	94	94	94

from each sample. However, the number of negative samples discovered out of the 94 listed for each of sample A2, B2, C2 and D2 agreed closely with the number expected from statistical estimates based on the MPN.

Comparison of BGA and BSA as media for subculture (Table 4) shows no significant difference. The use of only one BSA plate as opposed to two BGA plates may have influenced the results. With 12 samples BGA1 was positive whilst BGA2 was negative; with four samples BGA2 was positive when BGA1 was negative; with 149 samples both plates were positive.

When the results are examined on a laboratory rather than a sample basis agreement with the expected value does not seem as good (Table 5). It is possible that individual laboratories may have failed to use the technique efficiently but it is difficult to identify such laboratories from the results of this trial. Comparison of the findings on sample B2 and C2 which had broadly similar estimations of MPN suggests that the discrepancies were not due to variation between laboratories but to variation within the samples sent to each laboratory. Laboratories which found two positive tubes on sample B2 showed no tendency to do better than average on sample C2.

The variations in the concentration of organism within individual samples

Table 5. *Comparison of number of laboratories which isolated salmonellas from the different samples*

No. of laboratories	A2	B2	C2	D2
4	+	+	+	+
12	+	+	+	−
14	+	−	+	−
6	+	+	−	−
9	+	−	−	−
1	+	+	−	+
1	−	−	+	−
Total				
+ve	46	23	31	5
−ve	1	24	16	42
Expected number of laboratories negative for mean MPN/100 g based on counts	0·7	14·9	11·6	28·5

+ = at least 1 of 2 × 25 g tubes positive.

are more likely to have occurred at the time of their preparation and despatch than during transit, as postal conditions were likely to be similar for all samples.

Salmonella serotypes isolated from the test feed samples in the trial were *S. agona*, *S. anatum*, *S. cubana*, *S. derby*, *S. eimsbuettel*, *S. give*, *S. heidelberg*, *S. livingstone*, *S. muenster*, *S. newhaw*, *S. saintpaul*, *S. senftenberg*, *S. stanley*, *S. typhimurium* phage type 1a DT49, *S. virchow*, non-motile – O group B, non-motile – O group C.

DISCUSSION

The design of a standard technique for the isolation of *Salmonella* from animal feeds must take into account many factors such as food constituents, feed particle size, type and volume of pre-enrichment or selective media, plating media, incubation times and temperatures and methods of colonial identification. Many comparative exercises have been carried out to determine the optimum conditions and methods for isolating *Salmonella*, on a wide variety of materials. Carlson & Snoeyenbos (1974) indicated that tetrathionate was generally better than selenite for salmonella isolation regardless of the type of sample being cultured. Edel & Kampelmacher (1969, 1973, 1974) conducted trials using a standard technique which reduced the wide variability previously found between the methods of the participating laboratories. Smith & Moakes (in preparation) compared Muller Kauffman TB and selenite when used with BPW pre-enrichment, and also the value of BGA, BSA and DCLS. This work was the basis for the technique employed in these two trials.

In any trial intended to test the reproducibility of a technique it is important to limit the number of variable factors which may be introduced between the initial description and setting up of the technique and the final results as interpreted by the individual laboratory. In these studies such variables were controlled as fully as possible by issuing all media from one laboratory accompanied by

written instructions on their use. It was not possible to control humidity, the water used for reconstitution of media, the incubators used or the individual expertise of operators.

A standard technique of this type should be sensitive enough to detect *Salmonella* when present in small numbers. Very heavily contaminated test samples would probably be identified by every laboratory and this would not enable an assessment to be made of the sensitivity or true reproducibility of the method. When the concentration of organisms is low however, counts follow a Poisson distribution and not all the samples would be expected to be positive. The failure of laboratory H in the pilot trial to isolate salmonella from sample A1 may be due to the variation associated with the Poisson distribution, or to faulty media and methods.

The fact that 7 laboratories isolated *Salmonella* using the standard technique in the pilot trial (Table 1) is an indication that by carefully controlling variables it is possible to introduce a fairly new method to laboratories and yet achieve an acceptable reproducibility of results. The small number of laboratories involved and limited number of samples examined did not allow statistical analysis to be made, but a closer examination of the trial and its results indicated ways in which a more carefully controlled and extensive trial could be carried out.

The inoculation of two 9 cm diameter BGA plates as recommended by Edel & Kampelmacher (1969) allows better salmonella colonial differentiation than the use of a single plate. The method depends on the transfer of inoculum from the first to the second plate without intermediate sterilization of the inoculating loop. This allows for the dilution of heavily contaminated material and good separation of salmonella colonies. The advantage of inoculating two BGA plates is not easily identified from a single statistical appraisal of the number of plates showing growth but a general observation can be made that the use of two plates will aid the identification of colonies.

There is a significant inhibition of some salmonella serotypes by selective media (Carlson & Snoeyenbos, 1974). Sharma & Packer (1969) reported that *S. cholerasuis* would not grow in selenite and was tolerant to only low concentrations of brilliant green. The concentration of brilliant green in a plating medium is also critical for *S. dublin* and *S. typhimurium* (Magee & Hinton, 1974). McCoy (1962) stated that Rolfe's tetrathionate at 43 °C was lethal to most organisms including *Salmonella*.

It was felt that the BPW pre-enrichment stage used in the standard technique would encourage the multiplication of the relatively small numbers of salmonella in the samples including any sensitive serotypes which might be present thus enabling some of the increased numbers to survive the inhibitory effects of the MKTB medium. In individual instances a serotype was isolated from either BGA or BSA but no pattern was apparent from which it was possible to identify serotypes adversely affected by the media.

Smith & Moakes (in preparation) applied a standard technique to the monitoring of 469 feed samples for salmonella. The method involved the examination of duplicate units of 25 g from the test sample received in the laboratory. Of the

184 positive samples 51 (28%) would have been identified as negative if only a single unit of 25 g had been examined. The examination of more than two units of each sample may lead to an increase in isolations. The decision to examine single or multiple units of 25 g will relate to the number of samples being submitted from the bulk feed under test, the objectives of the sampling programme, the method by which the samples are obtained and the bacteriological level of acceptability in the food being tested.

The purpose of these trials has been to assess the reproducibility of a standard technique when variable factors were controlled to within reasonable limits.

A statistical comparison of expected and actual results shows that even when small numbers of salmonellas are present (MPN 2·3/100 g) the technique is capable of producing reproducible results.

Although this standard technique is recommended for the isolation of salmonellas from animal feeds, it should not discourage the use of additional methods where preferred, or when there is a special requirement for a more sensitive technique.

The grateful thanks of the Working Party are expressed to all laboratories that participated in these trials and, in particular, the laboratory staff at the Liverpool Veterinary Investigation Centre and Hull Public Health Laboratory who carried out the detailed preparation work. We are indebted to Miss C. N. Hebert of the Central Veterinary Laboratory who carried out the statistical analysis.

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APPENDIX 1. ISOLATION OF SALMONELLA FROM ANIMAL FEEDS INTER-LABORATORY TRIAL USING A STANDARD TECHNIQUE

1. Material and Methods

Food samples

To be issued from one laboratory. On receipt the 150 g samples should be stored at 4 °C until required for the examination which should commence on a Monday.

Media

Before receiving the test samples laboratories should familiarize themselves with methods of reconstituting the media and the colonial characteristics of a range of different organisms on the selective agar. Care should be taken that enough medium is left for the trial. All the media should be stored in a darkened cupboard until required. For the trial the media preparation should start on the Friday before the tests take place as indicated below.

2. Schedule for media preparation

Friday

(*a*) *Buffered Peptone Water* (*BPW*)

Peptone (Difco B118), 10 g; sodium chloride, 5 g; disodium hydrogen orthophosphate (12 H_2O) 9 g; potassium dihydrogen orthophosphate 1·5 g; deionized water 1 litre. Dissolve the above ingredients in the water by heating gently. Adjust the pH to 7·2. Distribute into 225 ml volumes in 1 lb honey jars or similar containers and sterilize at 15 lb/in^2 for 15 min.

(*b*) *Bismuth sulphite agar* (*BSA*) – *Lab M 13a and 13b**

BSA Base 'A'. Weigh out 18·2 g powder marked BSA 'A' and add to 500 ml deionized (DI) water, sterilize at 15 lb/in^2 for 15 min, cool to 55 °C and add 55 ml Chemical Mixture 'B'. Mix well and pour thin plates. Store at 4 °C for 3 days to mature before use.

BSA Chemical Mixture 'B'. Suspend 9 g of powder marked Chemical Mixture 'B' in 50 ml DI water. Bring to the boil over a tripod and gauze and cool quickly in cold water. Add to the 500 ml agar base.

Monday

(*a*) *Tetrathionate Broth Muller Kauffman* (*MKTB*) *Oxoid CM343*

Suspend 41 g of MKTB Base in 500 ml DI water. Bring to the boil and cool to 45 °C. Store in sealed flask at 4 °C. On day of use add iodine solution and brilliant green (see Tuesday).

(*b*) *Brilliant Green Agar* (*BGA*) – *Oxoid CM329*

Suspend 26 g of BGA in 500 ml DI water. Heat gently with occasional agitation and bring just to boil to dissolve. Cool to 55 °C, mix well and pour plates. *Do not autoclave.*

* Lab. M, London Analytical & Bacteriological Media Ltd, Pendleton, Lancs.

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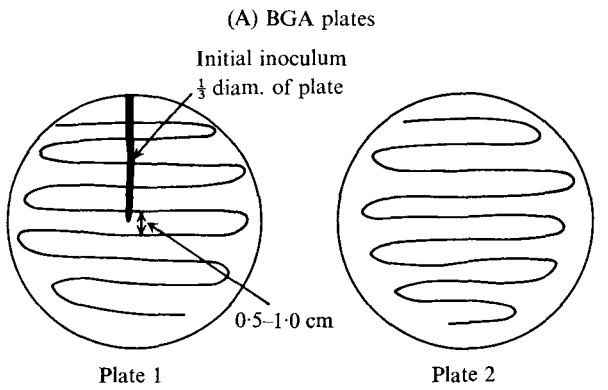

Fig. 1. Inoculation of plates.

Tuesday

Tetrathionate broth

Add 9·5 ml of iodine solution and 4·75 ml of 0·1 % brilliant green solution provided to the 500 ml of MKTB base prepared on Monday. Mix well and distribute in 100 ml volumes in 1 lb honey jars.

3. Examination of food samples

Monday

Remove bags of food from refrigerator at 9.00 a.m. and leave at room temperature. At 4.00 p.m. weigh out aseptically 2×25 g from each food sample and add each to a jar of 225 ml BPW .Incubate jars at 37 °C for 18 h.

Tuesday (10.00 a.m.)

Inoculate 10 ml from each jar of incubated BPW into a corresponding jar of 100 ml MKTB. Incubate the MKTB at 43 °C for 24 hours.

Wednesday (10.00 a.m.)

Plate out each MKTB on two plates (9 cm diam.) of BGA and a single plate of BSA using a 2·5 mm diameter loop. Take a droplet from the edge of the surface of the fluid. Inoculate the BGA plates according to Fig. 1A and the BSA plate according to Fig. 1B. Use the whole plate. Loop streaks should have spaces between each other of 0·5–1·0 cm. Use the same loop for all samples. Flame the loop between each different type of medium. Incubate at 37 °C overnight. Reincubate the MKTB for a further 24 h.

Thursday (9.00 a.m.)

(*a*) Examine plates. Select up to three colonies from any plate showing suspicious colonies and proceed to identify by subculture to composite media and by slide agglutination. It is suggested that either a composite medium which includes urea (eg. Kohn's) or triple sugar iron agar and a urea slope be inoculated with a portion of the suspect colony. Incubate the media at 37 °C overnight.

(*b*) Plate out the reincubated MKTB as previously described.

Friday (9.00 a.m.)

(*a*) Examine incubated composite media and record findings, discarding cultures which are obviously not *Salmonella*. Perform slide agglutination tests from the growth on the composite media using Wellcome Polyvalent 'O' and Polyvalent 'H' (phases 1, 2) salmonella agglutinating sera. If reactions occur with one or both sera perform as full examinations as possible and send a sub-culture to a reference laboratory for further typing.

(*b*) Examine plates and proceed as on Thursday.