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A rapid immunofluorescence technique for detecting salmonellae in raw meat

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INTRODUCTION

The sporadic presence of salmonellae in a wide range of food raw materials (Report, 1958; Hobbs & Wilson, 1959; Galbraith, Hobbs, Smith & Tomlinson, 1960; Kampelmacher, 1963) causes concern among food manufacturers and public health workers. Early detection of the contamination in such materials is one of the most important steps in preventing the spread of contamination and possible infection of the consumer. However, with existing techniques for salmonella detection the raw material could possibly be processed and distributed by the time the test answer is known. In addition, for many laboratories the existing techniques are so laborious that it is difficult to attain an adequate sampling rate. These difficulties have caused us to investigate means of radically shortening the salmonella test.

A salmonella detection technique should be able to detect contamination at least as low as one salmonella organism per 50 g. of sample (Hobbs, 1962). For this reason we have accepted that at this stage it is impossible to dispense with the liquid enrichment procedure, which is required to raise the salmonella numbers to a detectable level. However, 1 or 2 days would be saved if the salmonellae in enrichment broths could be demonstrated by direct examination, rather than by further culture on solid diagnostic media. On the basis of existing knowledge, the immunofluorescence technique (Beutner, 1961; Nairn, 1962) offered the best hope of such direct demonstration of salmonellae in enrichment cultures. Though the immunofluorescence technique by itself has not proved successful in detecting salmonellae in human stools (Thomason, Cherry & Edwards, 1959) success has been claimed for the technique in detecting *Salmonella dublin* in artificially contaminated milk (Arkhangel'skii & Kartashova, 1962). We hoped that with some adaptation it would prove suitable for the examination of naturally contaminated foods, particularly meats.

In early tests with pure cultures we found that salmonellae could be stained by the indirect immunofluorescence technique after culture in selenite broth at 43° C. (Plate 1A). We had already established experimentally that, as with faeces (Harvey & Thomson, 1953), enrichment at 43° C. in selenite F broth was very suitable for isolating salmonellae from foods (Georgala, 1963). Practical experience in our laboratory had further confirmed that 43° C. selenite enrichment was an excellent selective procedure for the examination of carcass and frozen boneless meats (to be published). We therefore decided that a rapid salmonella test could

possibly be based on the use of selenite enrichment for 18 hr. at 43° C., followed by immunofluorescence examination of the enrichment culture for the presence of salmonella cells. This communication describes our experiences with such a technique, during the examination of frozen imported meat samples.

The immunofluorescence examinations of the enrichments involved demonstrating O antigens only, as flagellar H antigens are very poorly developed in selenite broth at 43° C. The O antigens as demonstrated by immunofluorescence techniques are located in the cell wall, making the cell outline clearly visible (Thomason, Cherry & Moody, 1957). The indirect technique was used in our investigation because of the many different O antigens in the *Salmonella* group. In this way, only one fluorescent antiserum conjugate was required (e.g. a goat anti-rabbit globulin antiserum), which was then used in conjunction with various polyvalent or pure factor salmonella antisera produced in rabbits (Nairn, 1962). The indirect technique offered an added advantage, in that the first stage of the staining procedure could be performed with ordinary diagnostic salmonella agglutinating antisera.

The direct technique was also tried on a few occasions, and provided excellent staining of salmonellae grown in selenite broth. The staining procedure itself is shorter than the indirect technique, but the major drawback was the need for each polyvalent or single factor salmonella antiserum to be conjugated with fluorochrome.

METHODS AND MATERIALS

Meat samples

The frozen meat samples were obtained from the Food Hygiene Laboratory, Central Public Health Laboratory, Colindale. The samples were taken from shipments of imported frozen meat which had been shown at Colindale to be fairly heavily contaminated with salmonellae. In this way we hoped to avoid running into a long series of negative samples.

Reagents for indirect immunofluorescence testing

Salmonella antisera. Ordinary somatic diagnostic agglutinating antisera were used throughout as the first stage in the indirect immunofluorescence technique. These were obtained from the Standards Laboratory for Serological Reagents, Colindale, and when received had agglutination titres of around 1/250. For use in the indirect immunofluorescence technique the sera could be diluted up to one-quarter with phosphate buffered saline (pH 7·4). Most of the testing was done with the polyvalent O antiserum. However, the Standards Laboratory polyvalent antiserum contains only the most important O factors (Memorandum, 1961). In certain cases salmonellae were encountered which were not agglutinated or stained by this serum. The main example was *Salm. minnesota* (O:21) and to assist detection of this organism Burroughs Wellcome Salmonella O factor 21 serum was usually included in the staining procedure, as described later.

Preparation of fluorescent goat anti-rabbit serum. Rabbit globulin was prepared as follows. The blood of a number of exsanguinated rabbits was pooled, allowed to

stand overnight, and the serum collected and clarified by centrifuging. Using a saturated solution of ammonium sulphate, the serum was brought up to 40% ammonium sulphate saturation and left at room temperature for 1 hr. The precipitated globulins were centrifuged at 10,000 rev./min. for 20 min., and washed by centrifuging three times in fresh 40% saturated ammonium sulphate solution. The final sediment was redissolved in 20 ml. phosphate buffered saline (pH 7·4). This solution was then dialysed for 3 days at 4° C., against three changes of buffered saline. After dialysis the globulin solution was freeze dried, and the solid globulin stored at 4° C. The yield of dry globulins was 4·5 g. from 480 ml. serum.

To obtain an anti-rabbit antiserum, the rabbit globulin preparation was injected into a goat as follows:

	Period after 1st injection	Antigen and injection route
1st injection	0 weeks	500 mg. rabbit globulin dissolved in 8 ml. saline and mixed with 8 ml. complete Freund's adjuvant (Difco). Four intramuscular injections of 4 ml. each, one in each quarter
2nd injection	6 weeks	As above
3rd–7th injections	14 weeks	50 mg. rabbit globulin in 2 ml. saline intramuscularly on each of 5 consecutive days.
Bleeding	15 weeks	About 300 ml. blood drawn. Held at 4° C., and serum separated.

Conjugation of goat serum with fluorochrome. The methods used were identical with those described by Nairn (1962), and derived originally from Chadwick, McEntegart & Nairn (1958*a*, *b*) and Riggs *et al.* (1958). Lissamine rhodamine RB 200 (George Gurr Ltd) was normally used as fluorochrome, but an excellent fluorescent conjugate was also obtained using fluorescein isothiocyanate (Baltimore Biological Laboratories). In the latter case, the globulins in the serum were first precipitated with ammonium sulphate before conjugating them with the dye, thereby reducing the amount of the very expensive fluorochrome required. In addition fluorescein conjugates were purified by gel filtration through G. 25 Sephadex, instead of by treatment with activated charcoal as used for rhodamine conjugates (Nairn, 1962).

The goat anti-rabbit serum was prepared and conjugated in our laboratory so as to provide a large quantity of standardized reagent. However, similar anti-rabbit fluorescent sera are now available commercially and we have found that the Difco (U.S.A.) goat anti-rabbit fluorescein conjugate performs as well as our own serum.

Immunofluorescence testing of meat samples

The frozen meat samples were thawed, and then approximately 25 g. of each was cut into about a dozen small chunks and dropped into 100 ml. single strength selenite F broth (Leifson, 1936). If desired the sample could readily be increased to 50 g. in 200 ml. selenite broth. The enrichments were incubated for 18–24 hr.

in a 43° C. water-bath. A sample of each selenite enrichment was then withdrawn for immunofluorescence examination.

In the early stages of the work (first series of 158 samples) small quantities of the selenite enrichments were transferred with a small platinum loop to clean glass slides. The drops were smeared over about 0·25 cm.^{2}, air dried, and then fixed in formol saline (1 part formalin + 9 parts phosphate buffered saline pH 7·4) for at least 10 min. It was later found preferable to centrifuge (15 min. at 4000 rev./min.) 10 ml. quantities of the selenite broths, and resuspend the sediment in 0·5 ml. sterile distilled water (second series of 128 samples). Smears were then prepared from this suspension. The centrifuging had two advantages—smears of the distilled water suspensions were far easier to fix on the slides, and showed a concentration effect due to the change in total volume.

Fixed smears were washed in buffered saline, and freed of excess saline by passing rapidly through two baths of absolute alcohol, one bath of a 50:50 mixture of alcohol and xylene, and finally through a xylene bath before being air-dried. The dried fixed smears were covered with polyvalent salmonella O antiserum (duplicate smears were tested with O factor 21 antiserum if *Salm. minnesota* was expected) and held at room temperature in a Petri dish containing moistened filter paper to prevent evaporation of the serum. After 30 min. the excess salmonella antiserum was removed by washing in buffered saline, and the slides were dried as before. The smears were then covered with the goat anti-rabbit fluorescent conjugate, and held at room temperature for approximately 20 min. The slides were then washed in buffered saline, dried as before, and the smears mounted in D.P.X. mountant (G. Gurr Ltd) when rhodamine conjugate was used, or pH 7·4 buffered glycerol when fluorescein conjugate was used.

Microscopy

The Reichert fluorescence microscope was used, with an Osram HBO 200 mercury discharge lamp as lightsource. Most examinations were by brightfield illumination, with the condenser oiled to the underneath of the slide with liquid paraffin. A BG 12/4 mm. blue/ultra-violet exciter filter was used, in conjunction with an orange barrier filter. All smears were first examined with a × 40 dry fluorite objective, and doubtful cells checked with the × 100 oil immersion objective (Fluorite). Of several types of oil tested Microil (G. Gurr Ltd) was found to be the best liquid for use with the immersion objective. It possessed negligible fluorescence and was only slowly degraded by the intense ultra-violet illumination.

Assessment of stained smears

As the tests described in this report were of a preliminary nature, an arbitrary system of slide assessment was devised, based on the number of fluorescent cells seen in a preparation. Early experience had shown that one or two fluorescent cells in a whole smear did not usually indicate the presence of salmonellae. Such cells could be elements of the original flora, which had not multiplied during enrichment, and which cross-reacted with the fluorescent serum. On the basis of

known microscope field size, and the fairly constant volume/area ratio employed in smear preparation, it was decided that if salmonellae had multiplied significantly in the selenite enrichments at least one or more fluorescent cells would be visible in a field with the ×40 objective and ×8 ocular (Plate 1B, C). A sample was judged definitely free of salmonellae when its smear showed none or only a very few fluorescent cells over the whole area of the smear (0·25 cm.²). Samples providing smears with a fair number of fluorescent cells, but just not enough to average one or more per microscope field (×40 objective; ×8 ocular) were judged as probably containing salmonellae.

Confirmation of presence of salmonellae

After smears had been prepared from the 18–24 hr. selenite broth enrichments, each enrichment was also streaked on to Difco brilliant green agar (B.G.A.).

The enrichment tubes were incubated a further day at 43° C. and then again streaked on to fresh plates of B.G.A. The B.G.A. plates were incubated 18–24 hr. at 37° C. Suspicious pink colonies were identified as salmonellae or otherwise by biochemical and serological tests.

We have found the following tests most suitable for the rapid screening of colonies from B.G.A. plates:

(*a*) the lysine decarboxylase test of Møller (1955),

(*b*) the urease test on urea agar of Christensen (1946),

(*c*) the β-galactosidase test of Le Minor & Ben Hamida (1962).

Table 1 shows the reactions obtained with organisms which can produce pink colonies on B.G.A. plates.

Table 1. *Reactions used for screening organisms forming pink colonies on brilliant green agar*

	Lysine decarboxylase	Urease	β-Galactosidase
Salmonellae	+	−	− (Arizona +)
Not salmonellae (probably *Proteus*)	−	+	∓
Not salmonellae (probably paracolon)	−	∓	+

+, vigorous positive; ∓, usually negative, very occasionally weak positive.

Suspect salmonellae were typed by slide agglutination with polyvalent and single factor O and H salmonella antisera. Where flagellar phase changes were necessary we used the simple and very effective paper strip technique of Jameson (1961).

RESULTS

The results with the first series of 158 samples (no centrifuging) are shown in Tables 2 and 3. Table 2 shows that the fluorescence and conventional methods agreed exactly in the recording of *total* number of positive and negative samples. Table 3 reveals the actual agreements and discrepancies for individual samples, and shows that 80 % of the salmonella positive samples and 91 % of the salmonella negative samples were correctly identified by the fluorescence technique. There were ten false negative samples (fluorescence negative, conventional method positive) and ten false positives (fluorescence positive, conventional method negative). Serotypes isolated during these tests were: *Salm. minnesota, S. oranienburg, S. anatum, S. meleagridis, S. typhimurium, S. newport, S. paratyphi B, and S. orion.*

Table 2. *Detection of salmonella by fluorescence and conventional techniques in* 158 *frozen meat samples (first series, no centrifuging)*

	Fluorescence technique	Conventional technique
Samples positive	28 } Total 50	50
Samples probably positive	22 }	
Samples negative	108	108

Table 3. *Detailed comparison of results from* 158 *frozen meat samples examined by fluorescence and conventional techniques (first series, no centrifuging)*

Fluorescence technique	Conventional technique	No. of samples
Positive	Positive	24
Probably positive	Positive	16
Positive	Negative	4
Probably positive	Negative	6
Negative	Positive	10
Negative	Negative	98

In the second series of 128 samples (Tables 4 and 5) centrifuging of the selenite enrichment resulted in the detection by immunofluorescence of 87 % of the salmonella containing samples, as against 80 % in the first series of samples, i.e. a drop in the failure rate from 20 to 13 %. However, centrifuging also increased the false positive results from 6 to 12 % of the total samples examined.

Of the 13 % false negatives in the second series of samples, about half coincided with samples that required a full 48 hr. enrichment before salmonellae could be detected by conventional means. There seems to be little possibility that this slow development can be avoided—in our experience it occurs in about 5 or 6 % of the total positives encountered. The remaining false negatives represent definite failings in the immunofluorescence technique itself, e.g. inadequate fixing of the smears or incorrect assessment of doubtful slides. It seems likely that with more experience of the technique and with improved antisera these errors would be eliminated.

Table 4. *Detection of salmonella by fluorescence and conventional techniques in 128 meat samples (second series, selenite centrifuged)*

	Fluorescence technique		Conventional technique
Samples positive	50	Total 69	61
Samples probably positive	19		
Samples negative	59		67

Table 5. *Detailed comparison of results from 128 frozen meat samples examined by fluorescence and conventional techniques (second series, selenite centrifuged)*

Fluorescence technique	Conventional technique	No. of samples
Positive	Positive	47
Probably positive	Positive	6
Positive	Negative	3
Probably positive	Negative	13
Negative	Positive	8
Negative	Negative	51

DISCUSSION

The performance of the rapid immunofluorescence detection technique described here suggests that the technique might have applications in screening food raw materials for salmonellae. The serological cross-reactions were fewer than expected, possibly due to the selective enrichment procedure employed. Provided a number of samples are taken from each batch of meat there would usually be no difficulty in detecting batches that are considerably contaminated, or those that are completely free of salmonallae. It is lightly contaminated batches which could cause difficulty in interpretation, and here much would depend on the skill of the operator in assessing doubtful smears.

In its present form the 18 hr. immunofluorescence test would probably always miss those contaminated samples which require more than 24 hr. enrichment to be detected by conventional means. In our experience 43° C. selenite enrichment limits these to about 5 % of the total positives recorded, and any laboratory using the immunofluorescence technique would have to assess the importance of this level of failure. Other failures are possible but could probably be reduced by improved fixing of the smears, and consistent assessment of the stained preparations. Centrifuging of the selenite enrichments is preferred because it increased the detection rate, and assisted the fixing of the material on to the microscope slide. However, unless cross-reactions can be reduced by purifying the salmonella antisera, centrifuging would usually increase the rate of false positive results.

The immunofluorescence test described here depends on having a suitable polyvalent salmonella antiserum. Such an antiserum would have to include somatic antibodies for all the salmonella types expected in the materials being tested. In the present tests *Salm. minnesota* could not be detected with the polyvalent antiserum (which lacked O factor 21) and had to be tested for separately

on another smear. However, there should be no special difficulty in producing a range of polyvalent antisera to cover all known salmonella O group antigens.

It seems likely that some of the false positive results we recorded (fluorescence positive, conventional method negative) were due to non-specific cross-reactions, and could have been avoided by absorbing the antisera with selected non-salmonellae. A few suitable organisms were isolated and will be tested as absorbing agents. A few cross-reactions are due to organisms which possess antigens identical with those of salmonellae, and absorption is not likely to help with these.

An important advantage of the immunofluorescence technique described here is that the enrichment procedure is identical with the first stage of the conventional test for salmonellae. When smears have been prepared, the selenite enrichment can be streaked on a solid selective medium and the conventional technique proceeded with. Thus any batches of material recorded as doubtful positive by the immunofluorescence technique could be withheld from processing until this finding is confirmed the following day—the immunofluorescence technique would thus be used as a 'presumptive salmonella' test. Combined with an adequate sampling rate at an early enough stage such a 'presumptive test' could play a valuable role in preventing contaminated raw meats reaching the processing lines in food factories.

SUMMARY

A rapid 18 hr. technique has been developed for detecting salmonella contaminated carcass and boneless meats. It is based on 43° C. selenite enrichment of samples, followed by immunofluorescent detection of salmonella cells in the enrichment. In tests with 286 meat samples the rapid and conventional techniques agreed in the detection of 93 positive and 149 negative samples. The two tests failed to agree for the remaining 44 samples. The rapid technique thus lacks precision, but could be used as a rapid 'presumptive' salmonella test, so that contaminated material could be prevented from reaching the processing lines of food factories.

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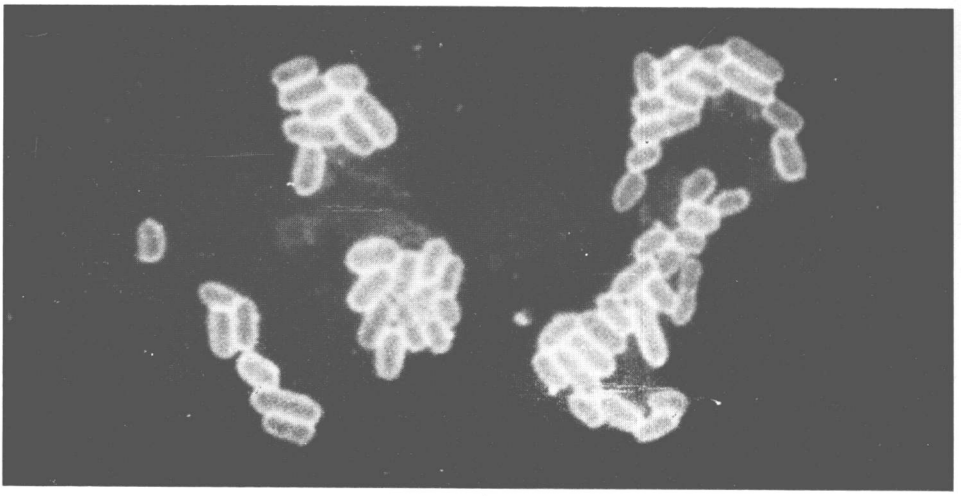

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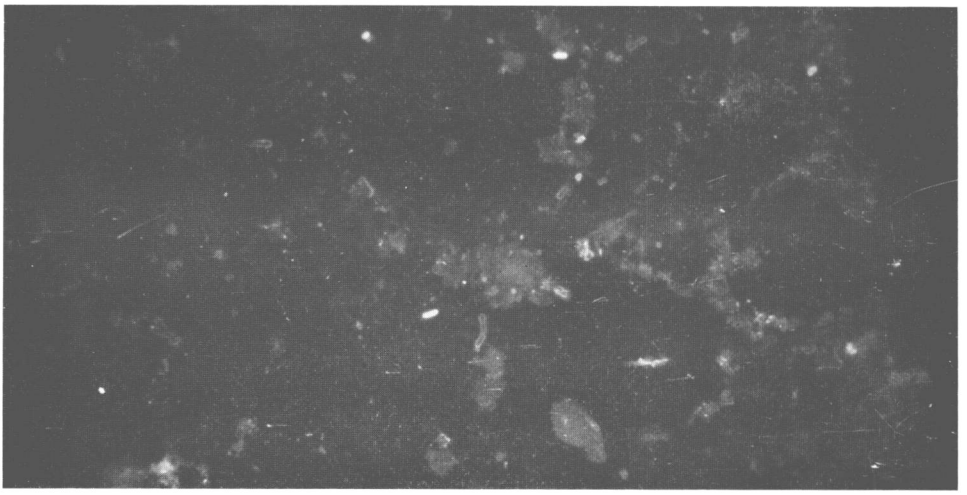

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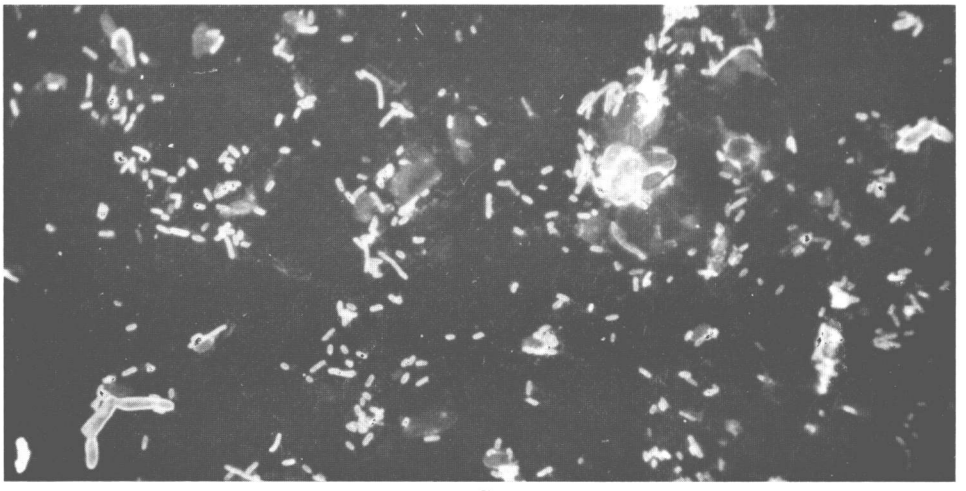

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EXPLANATION OF PLATE 1

A. *Salm. typhimurium* incubated 18 hr. in selenite broth at 43° C., then stained with Standards Laboratory polyvalent salmonella antiserum followed by rhodamine conjugated goat anti-rabbit antiserum. × 4000.

B. Smear prepared from a 18 hr. selenite broth enrichment of a contaminated meat sample; stained as for Plate 1A. Photographed with × 40 objective. A few strongly fluorescent salmonellae cells are visible amongst tissue debris and other bacteria. × 800.

C. Smear of sediment obtained from a centrifuged selenite enrichment of a contaminated meat sample; stained as for Plate 1A. This sample had provided a heavier growth of salmonellae than that shown in Plate 1B. × 800.