SCHEDULE 3 Regulation 21

Testing Methods

PART I

METHOD FOR THE ISOLATION OF *CLOSTRIDIUM PERFRINGENS*

Time of testing

1. Tests must be begun on receipt of the sample or on the first working day which allows this method to be completed. If the test is not begun on the day of receipt the sample must be stored in a refrigerator at between 2°C and 8°C until required. If the sample has been refrigerated it must be removed from the refrigerator and stored at room temperature for at least one hour before the test is started.

Samples

2. Tests must be carried out using two 10 gram portions of each sample submitted for testing. Each 10 gram sample must be placed aseptically in a sterile container containing 90 ml *Clostridium perfringens* diluent consisting of 0.1% peptone and 0.8% sodium chloride at a pH of 7 and mixed thoroughly until the sample is evenly suspended.

Inoculations

3. For each portion of the sample 1 ml of solution must be transferred to a sterile 90 mm petri dish (in duplicate), to which 15 ml of Shahidi – Ferguson agar (SF agar)(**1**) at a temperature of 47°C ±1°C must be added and immediately gently mixed by swirling the dish with 5 clockwise and 5 anticlockwise circular movements.

4. Once the agar has set, each agar plate must be overlaid with a further 10 ml SF agar at a temperature of 47°C±1°C. Once the overlay has set and with the plate lids uppermost the plates must be incubated anaerobically at 37°C±1°C for 20 hours±2 hours.

Samples with colonies of *Clostridium perfringens*

5. After incubation each set of duplicate plates must be examined for colonies characteristic of *Clostridium perfringens* (black). The sample provisionally fails if any colonies characteristic of *Clostridium perfringens* are present, in which case the following procedure must be followed to establish whether or not the colonies are *Clostridium perfringens*.

6. In the case of each plate, 10 characteristic colonies of *Clostridium perfringens* must be subcultured on to a further SF agar plate. If there are less than 10 colonies on the plate, all characteristic colonies must be subcultured on to the further plate. The plates must be incubated anaerobically at 37°C±1°C for 20 hours±2 hours.

7. If the surface area of the plates is overgrown and it is not possible to select well isolated characteristic colonies, 10 suspect colonies must be subcultured on to duplicate SF agar plates and incubated anaerobically at 37°C±1°C for 20 hours±2 hours.

8. One characteristic colony from each plate must be subcultured on to SF agar and incubated anaerobically at 37°C±1°C for 20 hours±2 hours.

(**1**) Shahidi-Ferguson agar – See Shahidi, S. A. and Ferguson, A. R. (1971) Applied Microbiology 21:500-506.American Society for Microbiology, 1913 1 St N.W., Washington DC 20006, USA.

Subcultured colonies

9. After incubation each plate must be examined for colonies characteristic of *Clostridium perfringens*. All colonies characteristic of *Clostridium perfringens* must be–

(a) stab inoculated into motility nitrate medium(**2**); and

(b) inoculated into either lactose gelatin medium(**3**) or charcoal gelatin discs(**4**);

and incubated anaerobically at 37°C±1°C for 20 hours±2 hours.

Motility

10. The motility nitrate medium must be examined for the type of growth along the stab line. If there is evidence of diffuse growth out into the medium away from the stab line, the bacteria must be considered to be motile.

Reduction of nitrate to nitrite

11. After examination of the motility nitrate medium, 0.2 ml to 0.5 ml of nitrite detection reagent must be added to it. The formation of a red colour confirms that the bacteria have reduced nitrate to nitrite. Cultures that show a faint reaction (i.e. a pink colour) should be discounted. If no red colour is formed within 15 minutes, a small amount of zinc dust must be added and the plate allowed to stand for 15 minutes. If a red colour is formed after the addition of zinc dust no reduction of nitrate to nitrite has taken place.

Production of gas and acid from lactose and liquefaction of gelatin

12. The lactose gelatin medium must be examined for the presence of small gas bubbles in the medium.

13. The lactose gelatin medium must be examined for colour. A yellow colour indicates fermentation of lactose.

14. The lactose gelatin medium must be chilled for one hour at 2 – 8°C and then checked to see if the gelatin has liquefied. If the medium has solidified it must be re-incubated anaerobically for a further 18 – 24 hours, the medium chilled for a further one hour at 2 – 8°C and again checked to see if the gelatin has liquefied.

15. The presence of *Clostridium perfringens* must be determined on the basis of the results from paragraphs 10 to 14. Bacteria which produce black colonies on SF agar, are non-motile, reduce nitrate to nitrite, produce gas and acid from lactose and liquefy gelatin within 48 hours must be considered to be *Clostridium perfringens*.

Control Tests

16. Control tests must be carried out each day that a test is initiated using–

(a) *Clostridium perfringens* no more than seven days old at the time of use;

(**2**) Motility nitrate medium – See Hauschild AHW, Gilbert RJ, Harmon SM, O"Keefe MF, Vahlefeld R, (1997) ICMSF Methods Study VIII, Canadian Journal of Microbiology 23, 884-892. National Research Council of Canada, Ottawa ON K1A oR6, Canada

(**3**) Lactose gelatin medium – See Hauschild AHW, Gilbert RJ, Harmon SM, O"Keefe MF, Vahlefield R, (1997) ICMSF Methods Study VIII, Canadian Journal of Microbiology 23, 884-892.

(**4**) Charcoal gelatin discs – See Mackie and McCartney, (1996) Practical Medical Microbiology 14, 509. Churchill Livingstone, Robert Stevenson House, 1-3 Baxter"s Place, Leith Walk, Edinburgh EH1 3AF.

(b) *Escherichia* coli NCTC 10418(**5**) or equivalent not more than seven days old at the time of use; and

(c) processed animal protein or compost or digestion residue which is free of *Clostridium perfringens*.

17. 10 gram portions of the rendered animal protein must be placed aseptically in each of two sterile containers containing 90 ml Buffered Peptone Water (BPW)(**6**) and mixed thoroughly until the samples are evenly suspended.

18. One colony of *Clostridium perfringens* must be placed in 10 ml BPW and mixed to form an even suspension. 0.1 ml of the suspension must be added to the suspension in the preceding paragraph. This must be repeated for *Escherichia* coli.

19. These are then treated and examined in the same way as test samples. If no typical colonies are formed then that day's testing must be invalid and must be repeated.

PART II

METHODS FOR THE ISOLATION OF SALMONELLA

A.

BACTERIOLOGICAL METHOD

1.—(1) Tests must be begun on receipt of the sample or on the first working day which allows this method to be completed. If the test is not begun on the day of receipt the sample must be stored in a refrigerator until required. If the sample has been refrigerated it must be removed from the refrigerator and stored at room temperature for at least four hours before the test is started.

(2) Tests must be carried out in duplicate using two 25 gram portions of each sample submitted for testing.

Day one

2. On day one, each 25 gram sample must be placed aseptically in a container containing 225 ml Buffered Peptone Water (BPW) and incubated at 37°C±1°C for 18 hours±2 hours.

Day two

3. On day two, 0.1 ml from the container of incubated BPW must be inoculated into 10 ml Rappaports Vassiliadis broth (RV broth)(**7**) and incubated at 41.5°C±0.5°C for 24 hours ± 3 hours.

Day three

4. On day three, the RV broth must be plated out on to two 90 millimetre plates of Brilliant Green Agar (BGA)(**8**) or on to one 90 millimetre plate of BGA and one 90 millimetre plate of Xylose

(**5**) The National Collection of Type Cultures, Central Public Health Laboratory, 61 Colindale Ave, London NW9 5HT.
(**6**) Buffered Peptone Water – See Edel, W. and Kampelmacher, E.H. (1973) Bulletin of World Health Organisation, 48: 167-174, World Health Organisation Distribution and Sales, CH-1211, Geneva 27, Switzerland (ISSN 0042-9686).
(**7**) Rappaports Vassiliadis Broth – See Vassiliadis P, Pateraki E, Papaiconomou N, Papadkis J A, and Trichopoulos D (1976) Annales de Microbiologie (Institut Pasteur) 127B: 195-200. Elsevier, 23 rue Linois, 75724 Paris, Cedex 15, France.
(**8**) Brilliant Green Agar – See Edel W and Kampelmacher E H (1969) Bulletin of World Health Organisation 41:297-306, World Health Organisation Distribution and Sales, CH-1211, Geneva 27, Switzerland (ISSN 0042-9686).

Lysine Deoxycholate Agar (XLD)(**9**) using a 2.5 mm diameter loop. The plates must be inoculated with a droplet taken from the edge of the surface of the fluid by drawing the loop over the whole of one plate in a zig zag pattern and continuing to the second plate without recharging the loop. The space between the loop streaks must be 0.5 cm – 1.0 cm. The plates must be incubated at 37°C ±2°C for 24 hours ± 3 hours.

5. The residual RV broth must be reincubated at 41.5°C±0.5°C for a further 24 hours.

Day four

6. On day four the plates must be examined and a minimum of 3 colonies from each plate showing suspicion of Salmonella growth must be subcultured–

(a) on to a blood agar plate;

(b) on to a MacConkey agar plate(**10**); and

(c) into biochemical media suitable for the identification of Salmonella.

These media must be incubated at 37°C overnight.

7. The reincubated RV broth must be plated out as described in paragraph 4.

Day five

8. On day five the incubated composite media or equivalent must be examined and the findings recorded, discarding cultures which are obviously not Salmonella. Slide serological tests must be performed using Salmonella polyvalent "O"and polyvalent "H" (phase 1 and 2) agglutinating sera on selected suspect colonies collected from the blood agar or MacConkey plates. If reactions occur with one or both sera, the colonies must be typed by slide serology. If requested in writing by the National Assembly , the operator of the laboratory must send a subculture to a Regional Veterinary Laboratory of the Veterinary Laboratories Agency of the Department for Environment, Food and Rural Affairs for further typing.

9. The plates referred to in paragraph 7 must be examined and further action taken as in paragraph 6 and 8.

B.

ELECTRICAL CONDUCTANCE METHOD

10. Tests must be begun on receipt of the sample or on the first working day which allows the following method to be completed. If the test is not begun on the day of receipt the sample must be stored in a refrigerator until required. If the sample has been refrigerated it must be stored at room temperature for at least four hours before the test is started.

Day one

11. On day one tests must be carried out in duplicate using two 25 gram portions of each sample submitted for testing. Each 25 gram sample must be placed aseptically in a sterile container

(**9**) Xylose Lisene Deoxycholate Agar – See Taylor W I, (1965) American Journal of Clinical Pathology, 44:471-475, Lippincott and Raven, 227E Washington Street, Philadelphia PA 19106, USA.

(**10**) MacConkey agar – See (1963) International Standards for Drinking Water, World Health Distribution and Sales, CH-1211, Geneva 27, Switzerland.

containing 225 ml Buffered Peptone Water/Lysine/Glucose (BPW/L/G)(**11**) and incubated at 37°C for 18 hours.

Day two

12. On day two the incubated BPW/L/G must be added to Selenite Cystine Trimethylamine-N-Oxide Dulcitol (SC/T/D)(**12**) and Lysine Decarboxylase Glucose (LD/G)(**13**) media in electrical conductance cells or wells. For cells or wells containing more than 5 ml medium 0.2 ml of the BPW/L/G must be added and for cells or wells containing 5 ml or less medium 0.1 ml of the BPW/L/G must be added. Cells or wells must be connected to appropriate electrical conductance measuring equipment set to monitor and record changes in electrical conductance at 6 minute intervals over a 24 hour period. The temperature of cells and wells must be kept at 37°C.

Day three

13. On day three, at the end of the 24 hour period, the information recorded by the conductance measuring equipment must be analysed and interpreted using criteria defined by the manufacturers of the equipment. Where a well or cell is provisionally identified as being positive for Salmonella, the result must be confirmed by subculturing the contents of the well or cell on to two 90 millimetre plates of BGA or on to one 90 millimetre plate of BGA and one 90 millimetre plate of Xylose Lysine Deoxycholate Agar (XLD) using a 2.5 mm diameter loop. The plates must be inoculated with a droplet taken from the edge of the surface of the fluid by drawing the loop over the whole of one plate in a zig zag pattern and continuing to the second plate without recharging the loop. The space between the loop streaks must be 0.5 cm – 1.0 cm. The plates must be incubated at 37°C overnight.

Day four

14. On day four the plates must be examined and a minimum of 3 colonies from each plate showing suspicion of Salmonella growth must be subcultured–

(a) on to a blood agar plate;

(b) on to a MacConkey agar plate; and

(c) into biochemical media suitable for the identification of Salmonella.

These media must be incubated at 37°C overnight.

Day five

15. On day five the incubated composite media or equivalent must be examined and the findings recorded, discarding cultures which are obviously not Salmonella. Slide serological tests must be performed using Salmonella polyvalent "O"and polyvalent "H" (phase 1 and 2) agglutinating sera on selected suspect colonies collected from the blood agar or MacConkey plates. If reactions occur with one or both sera, the colonies must be typed by slide serology. If requested in writing by the National Assembly , the operator of the laboratory must send a subculture to a Regional Veterinary Laboratory of the Veterinary Laboratories Agency of the Department for Environment, Food and Rural Affairs for further typing.

(**11**) Buffered Peptone Water/Lysine/Glucose – See Ogden I D (1988) International Journal of Food Microbiology 7:287-297, Elsevier Science BV, PO Box 211, 1000 AE, Amsterdam, Netherlands (ISSN 0168-1695).

(**12**) Selenite Cystine Trimethylamine-N-Oxide Dulcitol – See Easter, M C and Gibson, D M, (1985) Journal of Hygiene 94:245-262, Cambridge University Press, Cambridge.

(**13**) Lysine Decarboxylase Glucose- See Ogden I D (1988) International Journal of Food Microbiology 7:287-297, Elsevier Science BV, PO Box 211, 1000 AE, Amsterdam, Netherlands (ISSN 0168-1695).

PART III

METHOD FOR THE ISOLATION OF *ENTEROBACTERIACEAE*

1. Tests must be begun on receipt of the sample or on the first working day which allows this method to be completed. If the test is not begun on the day of receipt the sample must be stored in a refrigerator until required at between 2°C and 8°C. If the sample has been refrigerated it must be removed from the refrigerator and stored at room temperature for at least one hour before the test is started.

Samples

2. Tests must be carried out using five 10 gram portions of each sample submitted for testing. Each 10 gram sample must be placed aseptically in a sterile container containing 90 ml Buffered Peptone Water and mixed thoroughly until the sample is evenly suspended.

Inoculations

3. For each portion of the sample 1 ml of solution must be transferred to a sterile 90 mm petri dish (in duplicate). The plates must be labelled to identify the portion of sample they were taken from. 15 ml of Violet Red Bile Glucose Agar (VRBGA)(**14**) at a temperature of 47°C±2°C must be added to each petri dish and immediately gently mixed by swirling the dish with five clockwise and five anticlockwise circular movements.

4. Once the agar has set, each agar plate must be overlaid with a further 10 ml VRBGA at a temperature of 47°C±2°C. Once the overlay has set, the plates must be inverted and incubated aerobically at 37°C±1°C for 20 hours±2 hours.

Samples with colonies of *Enterobacteriaceae*

5. After incubation each set of duplicate plates must be examined for colonies characteristic of *Enterobacteriaceae* (purple colonies 1 – 2 mm in diameter). All characteristic colonies on each plate must be counted and the arithmetic mean of the duplicate plates taken.

The sample provisionally fails if either–

(a) any arithmetic mean is above 30(**15**); or

(b) three or more arithmetic means are above 10;

in which case the following procedure must be followed to establish whether or not the colonies are *Enterobacteriaceae*.

6. After counting the colonies, characteristic colonies must be taken at random from the agar plates, the number being at least the square root of the colonies counted. The colonies must be subcultured onto a blood agar plate and incubated aerobically at 37°C±1°C for 20 hours±2 hours.

Examination of subcultures

7. An oxidase test and a glucose fermentation test must be performed on each of the five subcultured colonies. Colonies which are oxidase-negative and glucose fermentation-positive must be considered to be *Enterobacteriaceae*.

(**14**) Violet Red Bile Glucose Agar – See Mossell D A A, Eelderink I, Koopmans M, van Rossem F (1978) Laboratory Practice 27 No. 12 1049-1050; Emap Maclaren, PO Box 109, Maclaren House, 19 Scarbrook Road, Croydon CR9 1QH.

(**15**) An arithmetic mean of 30 is equivalent to 3x102 colony forming units per gram of original sample.

8. If not all of the colonies prove to be *Enterobacteriaceae*, the total count in paragraph 5 must be reduced in proportion prior to establishing whether or not the sample should fail.

Controls

9. Control tests must be carried out each day that a test is initiated using–

(a) *Escherichia* coli NCTC 10418 no more than seven days old at time of use; and

(b) processed animal protein or compost or digestion residue which is free of *Enterobacteriaceae*.

10. A 10 gram portion of the rendered animal protein must be placed aseptically in a sterile container containing 90 ml BPW and mixed thoroughly until the sample is evenly suspended.

11. One colony of *Escherichia* coli must be placed in 10 ml BPW and mixed to form an even suspension. 0.1 ml of the suspension must be added to the suspension in the preceding paragraph.

12. This is then treated and examined in the same way as test samples. If no typical colonies are formed then that day's testing must be invalid and must be repeated.