SCHEDULE 2 Regulation 21

Testing Methods

PART 1

METHOD FOR THE ISOLATION OF *CLOSTRIDIUM PERFRINGENS*

Time of testing

1. Tests shall 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 shall be stored in a refrigerator at between 2°C and 8°C until required. If the sample has been refrigerated it shall be removed from the refrigerator and stored at room temperature for at least one hour before the test is started.

Samples

2. Tests shall be carried out using two 10 gram portions of each sample submitted for testing. Each 10 gram sample shall 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 shall 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 shall 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 shall 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 shall 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 shall 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 shall 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* shall be subcultured on to a further SF agar plate. If there are less than 10 colonies on the plate, all characteristic colonies shall be subcultured on to the further plate. The plates shall 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 shall 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 shall 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 shall be examined for colonies characteristic of *Clostridium perfringens*. All colonies characteristic of *Clostridium perfringens* shall 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.

EXAMINATION OF SUBCULTURES

Motility

10. The motility nitrate medium shall 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 shall 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 shall 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 shall 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 shall be examined for the presence of small gas bubbles in the medium.

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

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

15. The presence of *Clostridium perfringens* shall 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 shall be considered to be *Clostridium perfringens*.

Control Tests

16. Control tests shall 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) rendered animal protein which is free of *Clostridium perfringens*.

17. 10 gram portions of the rendered animal protein shall 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* shall be placed in 10 ml BPW and mixed to form an even suspension. 0.1 ml of the suspension shall be added to the suspension in the preceding paragraph. This shall 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 shall be invalid and shall be repeated.

PART 2

METHODS FOR THE ISOLATION OF *SALMONELLA*

A. BACTERIOLOGICAL METHOD

1. Tests shall 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 shall be stored in a refrigerator until required. If the sample has been refrigerated it shall be removed from the refrigerator and stored at room temperature for at least four hours before the test is started.

Day 1

2. Tests shall be carried out in duplicate using two 25 gram portions of each sample submitted for testing. Each 25 gram sample shall be placed aseptically in a container containing 225 ml Buffered Peptone Water (BPW) and incubated at 37°C for 18 hours±2 hours.

Day 2

3. 0.1 ml from the jar of incubated BPW shall be inoculated into 10 ml Rappaports Vassiliadis broth (RV broth)(**7**) and incubated at 41.5°C±0.5°C for 24 hours.

Day 3

4. The RV broth shall 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 Lysine Deoxycholate Agar (XLD)(**9**), using a 2.5 mm diameter loop. The plates shall 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 shall be 0.5 cm – 1.0 cm. The plates shall be incubated at 37°C±2°C for 24 hours ± 3 hours.

(**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).

(**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.

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

Day 4

6. The plates shall be examined and a minimum of 3 colonies from each plate showing suspicion of *Salmonella* growth shall 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 shall be incubated at 37°C overnight.

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

Day 5

8. The incubated composite media or equivalent shall be examined and the findings recorded, discarding cultures which are obviously not *Salmonella*. Slide serological tests shall 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 shall be typed by slide serology and a subculture sent to a veterinary laboratory nominated in writing by the Scottish Ministers for this purpose for further typing.

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

B. ELECTRICAL CONDUCTANCE METHOD

1. Tests shall 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 shall be stored in a refrigerator until required. If the sample has been refrigerated it shall be stored at room temperature for at least four hours before the test is started.

Day 1

2. Tests shall be carried out in duplicate using two 25 gram portions of each sample submitted for testing. Each 25 gram sample shall be placed aseptically in a sterile container containing 225 ml Buffered Peptone Water/Lysine/Glucose (BPW/L/G)(**11**) and incubated at 37°C for 18 hours.

Day 2

3. The incubated BPW/L/G shall 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 shall be added and for cells or wells containing 5 ml or less medium 0.1 ml of the BPW/L/G shall be added. Cells or wells shall be connected to appropriate electrical conductance measuring equipment set to

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

(**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).

monitor and record changes in electrical conductance at 6 minute intervals over a 24 hour period. The temperature of cells and wells shall be kept at 37°C.

Day 3

4. At the end of the 24 hour period, the information recorded by the conductance measuring equipment shall 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 shall 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 shall 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 shall be 0.5 cm – 1.0 cm. The plates shall be incubated at 37°C overnight.

Day 4

5. The plates shall be examined and a minimum of 3 colonies from each plate showing suspicion of *Salmonella* growth shall 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 shall be incubated at 37°C overnight.

Day 5

6. The incubated composite media or equivalent shall be examined and the findings recorded, discarding cultures which are obviously not *Salmonella*. Slide serological tests shall 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, a subculture shall be sent to a veterinary laboratory nominated in writing by the Scottish Ministers for this purpose for further typing.

PART 3

METHOD FOR THE ISOLATION OF *ENTEROBACTERIACEAE*

1. Tests shall 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 shall be stored in a refrigerator until required at between 2°C and 8°C. If the sample has been refrigerated it shall be removed from the refrigerator and stored at room temperature for at least one hour before the test is started.

Samples

2. Tests shall be carried out using five 10 gram portions of each sample submitted for testing. Each 10 gram sample shall 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 shall be transferred to a sterile 90 mm petri dish (in duplicate). The plates shall 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 shall 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 shall be overlaid with a further 10 ml VRBGA at a temperature of 47°C±2°C. Once the overlay has set, the plates shall 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 shall be examined for colonies characteristic of *Enterobacteriaceae* (purple colonies 1 – 2 mm in diameter). All characteristic colonies on each plate shall 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 shall be followed to establish whether or not the colonies are *Enterobacteriaceae*.

6. After counting the colonies, characteristic colonies shall be taken at random from the agar plates, the number being at least the square root of the colonies counted. The colonies shall 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 shall be performed on each of the five subcultured colonies. Colonies which are oxidase-negative and glucose fermentation-positive shall be considered to be *Enterobacteriaceae*.

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

Controls

9. Control tests shall 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 digestive residue which is free of *Enterobacteriaceae*.

10. A 10 gram portion of the rendered animal protein shall 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* shall be placed in 10 ml BPW and mixed to form an even suspension. 0.1 ml of the suspension shall 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 shall be invalid and shall be repeated.

(**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.