SCHEDULE 2 Regulation 21

TESTING METHODS

PART I

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 ± 1 gram portions of each sample submitted for testing. Each 10 gram ± 1 gram sample shall be placed aseptically in a sterile container containing 90 ml ± 1 ml *Clostridium perfringens* diluent consisting of 0.1% w/v peptone and 0.8% w/v, sodium chloride at a pH of 7.0 ± 0.2 and mixed thoroughly until the sample is evenly suspended.

Inoculations

3. For each portion of the sample 1 ml ± 0.1 ml of solution shall be transferred to a sterile 90 mm petri dish (in duplicate), to which 15 ml ± 1 ml of Egg-yolk-free Tryptose-Sulphite-Cycloserine agar (EY-free TSC agar)(**1**) at a temperature of 46°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 EY-free TSC agar at a temperature of 46°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 containing well separated colonies, 3 characteristic colonies of Clostridium perfringens shall each be subcultured onto a further EY-free TSC agar plate. If there are less than 3 colonies on the plate, all characteristic colonies shall be subcultured onto further plates. 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, an attempt shall be made to subculture 3 suspect colonies onto duplicate EY-free TSC agar plates and incubated anaerobically at 37°C ± 1°C for 20 hours ± 2 hours. Subsequent purification may be required in order to obtain well isolated colonies of suspect organism.

(**1**) E-Y free TSC agar – *See* Hauschild, and Hilsheimer, R (1974) Applied Microbiology 27: 78-82

8. One characteristic colony from each plate shall be subcultured onto EY-free TSC agar and incubated anaerobically at 37°C ± 1°C for 20 hours ± 2 hours.

Subcultured colonies

9. After incubation each plate shall be examined for colonies characteristic of *Clostridium perfringens*. At least 3 colonies characteristic of *Clostridium perfringens* shall be –

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

(b) inoculated into either lactose gelatine medium(**3**)

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 tube allowed to stand for approximately15 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 gelatine

12. The lactose gelatine medium shall be examined for the presence of small gas bubbles in the medium.

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

14. The lactose gelatine medium shall be chilled for up to one hour at 2–8°C and then checked to see if the gelatine has liquefied. If the medium has solidified it shall 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 gelatine 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 EY-free TSC agar, are non-motile, reduce nitrate to nitrite, produce gas and acid from lactose and liquefy gelatine 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 –

(**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 gelatine medium – *See* Hauschild AHW, Gilbert R J, Harmon S M, O'Keefe MF, Vahlefeld R, (1997) ICMSF Methods Study VIII, Canadian Journal of Microbiology 23, 884-892

(a) *Clostridium perfringens*NCTC 10662(**4**) no more than 28 days old at the time of use;

(b) *Escherichia coli* NCTC 10418 or equivalent not more than 28 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 ± 1 gram portions of the rendered animal protein shall be placed aseptically in each of two sterile containers containing 90 ml ± 1 ml Buffered Peptone Water (BPW)(**5**) and mixed thoroughly until the samples are evenly suspended.

18. One colony of *Clostridium perfringens* (16)(a) shall be placed in 10 ml ± 1 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* (16)(b).

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 II

METHODS FOR THE ISOLATION OF*salmonella*

A. BACTERIOLOGICAL METHOD

20. 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 one hour before the test is started.

Day 1

21. Tests shall be carried out in duplicate using two 25 gram ± 1 gram portions of each sample submitted for testing. Each 25 gram ± 1 gram sample shall be placed aseptically in a container containing 225 ml ± 1 ml Buffered Peptone Water (BPW) and incubated at 37°C ± 1°C for 18 hours ± 2 hours.

Control Tests

22. Control tests shall be carried out each day that a test is initiated using –

(a) *Salmonella java* NCTC 5706 or equivalent no more than 28 days old at the time of use;

(b) *Erwinia herbicola* NCTC 9381 or equivalent not more than 28 days old at the time of use; and

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

23. 10 gram ± 1 gram portions of the rendered animal protein shall be placed aseptically in each of two sterile containers containing 90 ml ± 1 ml Buffered Peptone Water (BPW)(**6**) and mixed thoroughly until the samples are evenly suspended.

(**4**) The National Collection of Type Cultures, Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT.

(**5**) 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)

(**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)

24. One colony of *Salmonella java* (22)(a) shall be placed in 10 ml ± 1 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 *Erwinia herbicola* (22)(b).

25. 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.

Day 2

26. 0.1 ml from the container of incubated BPW shall be inoculated into 10 ml ± 1 ml Rappaport-Vassiliadis Soya Broth (RVS broth)(**7**) and incubated at 41.5°C ± 0.5°C for 18 to 24 hours.

Day 3

27. The RVS broth shall be plated out onto two 90 mm plates of Brilliant Green Agar (BGA)(**8**) or onto one 90 mm plate of BGA and one 90 mm 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 ± 1°C for 18 to 24 hours.

28. The residual RVS broth shall be reincubated at 41.5°C ± 0.5°C for a further 18 to 24 hours.

Day 4

29. The plates shall be examined and a minimum of 3 colonies from each plate showing suspicion of *Salmonella* growth shall be subcultured –

(a) onto a nutrient agar plate;

(b) onto a MacConkey agar plate(**10**); and

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

These media shall be incubated at 37°C ± 1°C overnight.

30. The reincubated RVS broth shall be plated out as described in paragraph 27.

Day 5

31. 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 nutrient agar or MacConkey agar plates. If reactions occur with one or both sera, a subculture of the colonies shall be sent to one of the Department's laboratories at either Agriculture, Food and Environmental Science Division, Newforge Lane, Belfast, BT9 5PX or, Veterinary Science Division, Stoney Road, Belfast, BT4 3SD, for further typing.

32. The plates referred to in paragraph 30 shall be examined and further action taken as in paragraphs 29 and 31.

(**7**) Van Schothurst M., Renauld A., and Van Beek C. (1987) Food Microbiology 4: 11-18.

(**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 Lysine Deoxycholate Agar – *See* Taylor, W.I. (1965) American Journal of Clinical Pathology, 44:471-475, Lippincott and Raven, 227 E. Washington Street Philadelphia PA19106, USA

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

B. ELECTRICAL CONDUCTANCE METHOD

33. Tests shall be begun on receipt of the samples 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 removed from the refrigerator and stored at room temperature for at least one hour before the test is started.

Day 1

34. Tests shall be carried out in duplicate using two 25 gram ± 1 gram portions of each sample submitted for testing. Each 25 gram ± 1gram sample shall be placed aseptically in a sterile container containing 225 ml ± 1 ml Buffered Peptone Water (BPW) (**11**) and incubated at 37°C ± 1°C for 18 to 24 hours.

Control Tests

35. Control tests shall be carried out each day that a test is initiated using –

(a) *Salmonella java* NCTC 5706 or equivalent no more than 28 days old at the time of use;

(b) *Erwinia herbicola* NCTC 9381 or equivalent not more than 28 days old at the time of use; and

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

36. 10 gram ± 1 gram portions of the rendered animal protein shall be placed aseptically in each of two sterile containers containing 90 ml ± 1 ml Buffered Peptone Water (BPW)(**11**) and mixed thoroughly until the samples are evenly suspended.

37. One colony of *Salmonella java* (35)(a) shall be placed in 10 ml ± 1 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 *Erwinia herbicola* (35)(b).

38. 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.

Day 2

39. The incubated BPW shall be added to Rappaport -Vassiliadis Soya (RVS) Broth in tubes to be inserted into electrical conductance cells. Detection of growth will utilise indirect impediometry as in the method of Donaghy and Madden (1993)(**12**). For cells or tubes containing more than 5 ml (RVS) medium 0.2 ml of the BPW shall be added and for cells or tubes containing 5 ml or less (RVS) medium 0.1 ml of the BPW shall be added. Cells or tubes shall 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 tubes shall be kept at 42°C ± 0.5°C.

Day 3

40. 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 tube or cell is provisionally identified as being positive for *Salmonella*, the result shall be confirmed by subculturing the contents of the tube or cell onto two 90 mm plates of

(**11**) Buffered Peptone Water – *See* Edel, W. and Kampelmacher, E. H. (1973) Bulletin of World Health Organisation, 167-174, World Health Organisation Distribution and Sales, CH-1211, Geneva 27, Switzerland (ISSN 0042-9686)

(**11**) Buffered Peptone Water – *See* Edel, W. and Kampelmacher, E. H. (1973) Bulletin of World Health Organisation, 167-174, World Health Organisation Distribution and Sales, CH-1211, Geneva 27, Switzerland (ISSN 0042-9686)

(**12**) Donaghy and Madden – *See* Donaghy, J. A. and Madden R. H. (1993) International Journal of Food Microbiology. 17; 281-288

BGA or onto one 90 mm plate of BGA and one 90 mm 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 ± 1°C overnight.

Day 4

41. The plates shall be examined and a minimum of 3 colonies from each plate showing suspicion of *Salmonella* growth shall be subcultured –

(a) onto a nutrient agar plate;

(b) onto a MacConkey agar plate; and

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

These media shall be incubated at 37°C ± 1°C overnight.

Day 5

42. 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 nutrient agar or MacConkey agar plates. If reactions occur with one or both sera, a subculture of the colonies shall be sent to one of the Department's laboratories at either Agriculture, Food and Environmental Science Division, Newforge Lane, Belfast, BT9 5PX, or Veterinary Science Division, Stoney Road, Belfast, BT4 3SD, for further typing.

PART III

METHOD FOR THE ISOLATION OF*enterobacteriaceae*

43. 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

44. Tests shall be carried out using five 10 gram ± 1 gram portions of each sample submitted for testing. Each 10 gram ± 1 gram sample shall be placed aseptically in a sterile container containing 90 ml ± 1 ml Buffered Peptone Water (BPW) and mixed thoroughly until the sample is evenly suspended.

Controls

45. Control tests shall be carried out each day that a test is initiated using –

(a) *Escherichia coli* NCTC 10418 no more than 28 days old at time of use; and

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

46. A 10 gram ± 1 gram portion of the rendered animal protein shall be placed aseptically in a sterile container containing 90 ml ± 1 ml BPW and mixed thoroughly until the sample is evenly suspended.

47. One colony of *Escherichia coli* shall be placed in 10 ml ± 1 ml BPW and mixed to form an even suspension. Approximately 0.1 ml of the suspension shall be added to the suspension in the preceding paragraph.

48. 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.

Inoculations

49. For each portion of the sample 1 ml ± 0.1 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 ± 1 ml of Violet Red Bile Glucose Agar (VRBGA)(**13**) at a temperature of 46°C ± 1°C shall be added to each petri dish and immediately gently mixed by swirling the dish with five clockwise and five anticlockwise circular movements.

50. Once the agar has set, each agar plate shall be overlaid with a further 10 ml (approximately) of VRBGA at a temperature of 46°C ± 1°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*

51. 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(**14**); 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*.

52. 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. Each of the colonies shall be subcultured onto a nutrient agar plate and incubated aerobically at 37°C ± 1°C for 20 hours ± 2 hours.

Examination of subcultures

53. 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*.

54. If not all of the colonies prove to be *Enterobacteriaceae*, the total count in paragraph 51 shall be reduced in proportion prior to establishing whether or not the sample should fail.

(**13**) Violet Red Bile Glucose Agar – *See* Mossel, 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.

(**14**) An arithmetic mean of 30 is equivalent to 3×10^2 colony forming units per gramme of original sample