SCHEDULE 3

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.

(**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).