SCHEDULE 2

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.

(**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)