Council Directive 93/85/EEC of 4 October 1993 on the control of potato ring rot

ANNEX I

METHOD FOR THE DETECTION AND DIAGNOSIS OF THE RING ROT BACTERIUM, CLAVIBACTER MICHIGANENSIS (Smith) Davis et al. ssp. SEPEDONICUS (Spieckermann et Kotthof) Davis et al. IN BATCHES OF POTATO TUBERS

5. **Scheme for IF-testing**

5.1. Use antiserum to a known strain of *C. sepedonicum* — ATCC 33113 (NCPPB 2137), or NCPPB 2140. This should have an IF titre of more than 1:600. Include one PBS control on the test slide to determine whether the fluorescein isothiocyanate anti-rabbit immunoglobulin conjugate (FITC) combines non-specifically with bacterial cells. *Corynebacterium sepedonicum* (ATCC 33113 (NCPPB 2137), NCPPB 2140) should be used as homologous antigen controls on a separate slide. Naturally infected tissue (maintained by lyophilization or freezing at - 20°C) should be used where possible as a similar control on the same slide (Figure 2).

5.2. *Procedure*

5.2.1. Prepare three serial ten fold dilutions (10^1, 10^2, 10^3) of the final pellet in distilled water (Figure 1).

5.2.2. Pipette a measured standard volume sufficient to cover the window (approximately 25 µl) of each pellet dilution or *C. sepedonicum* suspension (approximately 10^6 cells/ml) to windows of a multispot slide as shown in Figure 1.

Figure 1

Sample and PBS control slide

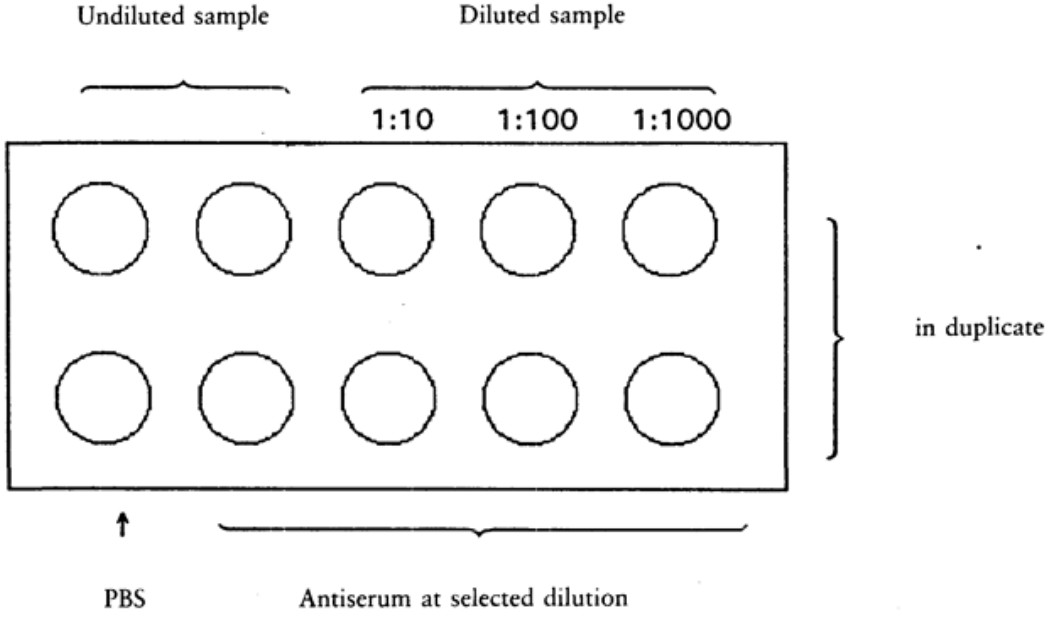
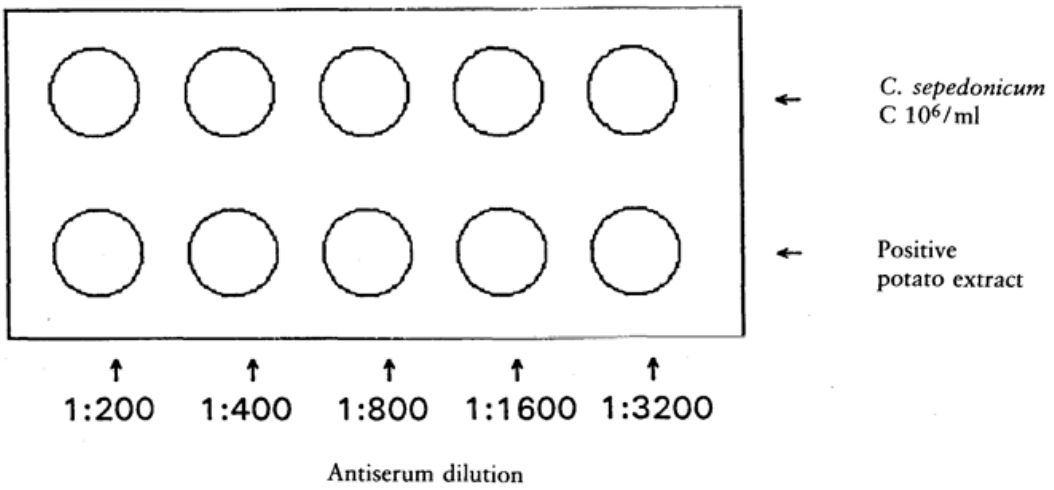


Figure 2

Positive control slide



5.2.4. Cover appropriate windows which *C. sepedonicum* antiserum at the recommended dilutions, 0,01 M PBS pH 7,2 (Appendix 2), as shown in Figure 1. (Use PBS for the FITC control.) The working dilution of the antiserum should be approximately half that of the IF titre. If other antiserum dilutions are to be included, separate slides should be prepared for each dilution to be used.

5.2.5. Incubate in a humid chamber at ambient temperature for 30 minutes.

5.2.6. Rinse carefully with 0,01 M PBS pH 7,2. Wash for five minutes in three changes of 0,01 M PBS pH 7,2.

5.2.7. Carefully remove excess moisture.

5.2.8. Cover each window with FITC conjugate at the same dilution used to determine the titre and incubate in a dark humid chamber at ambient temperature for 30 minutes.

5.2.9. Rinse and wash as before.

5.2.10. Apply approximately 5 to 10 µl of 0,1 M phosphate buffered glycerine pH 7,6 (or a similar mountant with a pH not less than 7,6) to each window and cover with a coverglass (Appendix 2).

5.2.11. Examine with a microscope fitted with an epifluorescent light source and filters suitable for working with FITC. A magnification of 400 to 1 000 is suitable. Scan replicated windows across two diameters at right angles and around the window perimeters.

Observe for fluorescing cells in the positive controls and determine the titre. Observe for fluorescing cells in the FITC/PBS control window and, if absent, proceed to the test windows. Determine in a minimum of 10 microscope fields the mean number of morphologically typical fluorescing cells per field and calculate the number per ml of undiluted pellet (Appendix 4).

There are several problems inherent to the immunofluorescence test.

— Background populations of fluorescing cells with atypical morphology and cross reacting saprophytic bacteria with size and morphology similar to *Clavibacter michiganensis* ssp. *sepedonicus* are likely to occur in potato pellets. Consider only fluorescing cells with typical size and morphology.

Because of the possibility of cross-reactions, samples with a positive immunoflorescence test should be retested using a different antiserum.

— The technical limit of detection of this method is situated between 10^3 and 10^4 cells per ml of undiluted pellet. Samples with counts of IF typical cells at the detection limit are usually negative for C. *m.* ssp. *sepedonicus* but may be committed to eggplant testing.

A negative immunofluorescence test is identified for any sample where morphologically typical fluorescing cells are not found. The samples shall be considered as *'not contaminated'* with *Clavibacter michiganensis* ssp. *sepedonicus*.

The eggplant test is not required.

A positive immunofluorescence test is identified for any sample where morphologically typical fluorescing cells are found.

Samples for which a positive immunofluorescence test have been identified with both antisera shall be considered as *'potentially contaminated'* with *Clavibacter michiganensis* ssp. *sepedonicus*.

The eggplant test is required for all samples considered as potentially contaminated.