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BIO-FLUO IHN (BIO K 008)

IMMUNOFLUORESCENT ASSAY FOR THE DETECTION OF INFECTIOUS HAEMATOPOIETIC NECROSIS (IHN) VIRUS IN CELL CULTURES

I - INTRODUCTION

Infectious haematopoietic necrosis (IHN) was until recently confined mainly to the North American Pacific Coast and certain countries of the Far East. The isolation of this virus in two French trout farms in the spring of 1987 (de Kinkelin et al., 1987) revealed the existence of this virus in Europe. Since then, other foci have been described in a series of countries. This viral infection is caused by a rhabdovirus. There are currently 4 or 5 known subgroups of this virus (Hattenberger and de Kinkelin, 1988). Several Salmonidae species are sensitive to the virus. They include various Pacific salmon species (Onchorhynchus sp.), the Atlantic salmon (Salmo salar) and the rainbow trout (Onchorhynchus mikiss). The brown trout (Salmo trutta fario) has recently been shown to be sensitive to the virus (La Patra and Fryer, 1990). Pike fry (Esox lucius) is sensitive to the virus experimentally (Dorson et al., 1987). The clinical disease generally occurs in water at temperature below 14° C. It is characterized by nervous system and digestive disordres: alternating apathy and spasmodic movements and enteritis as evidenced by long, whitish excrement. Autopsy reveals exophtalmia, ascites and haemorrhages in the muscle mass and viscera (de Kinkelin, 1970). The mortality rates associated with the virus can be high. It is almost impossible to distinguish IHN from viral haemorrhagic septicaemia (VHS), another Salmonidae viral infection likewise caused by a rhabdovirus, on the basis of clinical evidence alone. A differential diagnosis obtained by laboratory investigation thus appears to be indispensable. While neutralizing antibodies have been detected (Hattenberger et al., 1989), confirmation of the clinical diagnosis usually relies on isolation of the virus on a cell culture.

II - PRINCIPLE OF THE TEST

The infected specimens are ground up in a mortar with the help of sand, then put in solution in an antibiotic-supplemented culture medium. The preparation is centrifuged and a 24-well cell culture plate inoculated with a serial dilution of the supernatant. After 1 hour's incubation at optimal temperature culture medium is added to each well and the plate is incubated until a cytopathogenic effect is observed. At this point, the cell preparation is fixed, then rinsed. IHN-specific monoclonal antibody is then added and the plate returned to the incubator. After this first incubation with monoclonal antibody the plate is rinsed, then the conjugate, goat anti-mouse FITC (fluorescein-coupled mouse-immunoglobulin-specific goat immunoglobulin) is added to each well and the plate incubated once more. The plate is then rinsed, mounting medium added to each well, and the cell layer observed under an inverted microscope equipped for fluorescence. If the virus causing infectious haematopoietic necrosis is present, a green color will be seen at the sites of viral replication.

III - COMPOSITION OF THE KIT

- 1 X 100-ml bottle of washing solution: 10-fold concentrate of PBS to be diluted in 900 ml of distilled water: washing solution.
- 1 X 25-ml bottle of fixative (ready-to-use acetone solution): fixation solution.
- 1 bottle containing monoclonal antibody specific for IHN virus: IHN-specific monoclonal antibody.
- 1 bottle of fluorescein-coupled anti-mouse-immunoglobulin conjugate: Goat anti Mouse Ig FITC Conjugate.
- 1 X 25-ml bottle of an Evan's blue-containing buffer solution for diluting the conjugate: dilution solution.
- 1 X 25-ml bottle of mounting medium: mounting medium.

IV - PRECAUTIONS FOR USE

- This test may be used for in vitro diagnosis only.
- The reagents must be stored between +2°C and +8° C; they may be used up until the shelf-life date on the package.
- Do not use reagents from other kits.
- Discard solutions contaminated by bacteria or fungi.
- Some bottles contain sodium azide. This product is toxic if inhaled or if it comes in contact with the skin. Take the usual precautions when handling these bottles.
- Avoid all risks of environmental contamination by inactivating all solutions likely to contain viruses that are pathogenic for fish with a 2% sodium hypochlorite solution.

V – PROCEDURE

For countries belonging to the European Communities, sampling plans and diagnostic methods for the detection and confirmation of viral haemorrhagic septicaemia (VHS) and infectious haematopoietic necrosis (IHN) must be applied (2001/183/EC) – Commission decision of 22 February 2001.

1. Extracting the virus

1.1. Preparing the specimens

Take from moribund fish or fresh corpses approximately 1-gram fragments of spleen, kidney and brain tissue. Mix these fragments with oven-sterilised sand and grind the mixture in a mortar. After complete homogenisation is achieved add 2 ml of culture medium containing 2% foetal calf serum and antibiotics (inoculation medium). For example, one may use a mixture of 200 IU of penicillin, 200 µg of streptomycin and 200 µg of kanamycin per ml of culture medium. A ready-to-use antibiotic + antifungal mixture that has been optimised for this purpose is available from Bio-X. This mixture avoids the problems encountered when cell cultures are inoculated with heavily-contaminated specimens. For small fish, the entire corpse may be homogenised in the mortar, ideally after the intestines have been resected.

1.2. Centrifugation of specimens

The homogenised preparation is centrifuged at between 2000 and 4,000 g at 4° C for 15 minutes. The supernatant is collected for the subsequent steps.

1.3. Dilution of specimens

1:10, 1:100 and 1:1,000 dilutions of the supernatant are made using the inoculation medium.

2. Isolating the virus

2.1. Cell line selection

The FHM, EPC, BF.2 and RTG2 cell lines are susceptible to IHN virus. These cells may be grown in Eagle's modified MEM or with better results in Glasgow's MEM supplemented with 10% foetal calf serum, 10% phosphate tryptose and a mixture of antibiotics at the standard concentration. If a CO_2 incubator is not available, the medium may be buffered at pH 7.4 with 0.16 M Tris-HCl. The optimal temperature for growth is 30° C for the FHM and EPC cells, 25° C for the BF.2 cells and 21° C for the RTG2 cells (de Kinkelin et al., 1986).

2.2. Preparation of the cellular substrate

The cells are kept in a Roux flask at their optimal growth temperature. One to two days before use the cells are treated with trypsin to separate them from their backing, then seeded on a 24-well plate. As susceptibility to the virus depends on the cells' age, it is advisable to use them 24-48 hours after their transfer to the plate. To guarantee the quality of the diagnosis, the cell layer must be in perfect condition at the time of inoculation with the specimens.

2.3. Inoculation

The culture medium is eliminated by turning the plate upside down over a receptacle. Use a sharp movement so as to avoid adsorption of the culture medium on the outer surface of the well. In carrying out this step, hold the plate at a reasonable distance from the receptacle to avoid all risks of contamination from splashes. After emptying the plate, quickly deposit the different dilutions of specimens, for the cell layer must be kept moist at all times. The specimens must be deposited in the wells very delicately so as not to damage the cell layer. If automatic microtip pipettes or Pasteur pipettes are used, place the tip of the pipette against the side wall of the well and release the sample material slowly. 200 µl aliquots of the different dilutions are placed in each well. Incubate the plate at 15° C for 1 hour.

2.4. Addition of inoculation medium

At the end of the viral adsorption period add gently to each well 1 ml of the 2% foetal calf serum culture medium (inoculation medium).

2.5. Incubating the plate

The plate is kept in an incubator (under 5% CO_2) at the optimum temperature for viral growth (15° C). It is inspected daily until a cytopathogenic effect is observed. This consists of the development of dense, spherical cells, the destruction of which results in the formation of plaques.

3. Identifying the virus

3.1. Fixation

When the cytopathogenic effect is clearly visible, which happens, for example, in the case of infectious haematopoietic necrosis virus, in 48-72 hours, the cell layer is fixed with the acetone solution. Eliminate the culture medium by turning the plate upside down, then add, gently, 500 µl of fixative per well. Special care must be taken to avoid adding liquid to the wells violently or letting the preparation dry out during the various steps for revealing the virus, for the cell layer could separate from the well. Incubate the plate at 4° C

for 20 minutes, then eliminate the fixative by turning the plate upside down and placing it, face down, on absorbent paper for a few seconds.

3.2. Washing the plate

Add delicately 1 ml of a 1:10 dilution of the concentrated washing solution to each well. Wait a few minutes, then remove the washing solution by turning the plate over. Repeat this operation, then place the plate face down on absorbent paper.

3.3. Addition of monoclonal antibody

Just before starting the test, dilute the mother solution twentyfold with a 1:10 dilution of the concentrated washing solution. Add 200 µl of the diluted monoclonal antibody solution to each well and incubate the plate + 21°C +/- 3°C for 1 hour. Unused and undiluted monoclonal antibody is to be stored between +2°C and +8°C.

3.4. Washing the plate

Proceed as described above in point 3.2.

3.5. Adding the conjugate

Just before starting the test, dilute the mother solution twentyfold with the conjugate dilution solution. Add 200 µl of the diluted conjugate to each well and incubate the plate + 21°C +/- 3°C for 1 hour. Unused and undiluted conjugate is to be stored between +2°C and +8°C.

3.6. Washing the plate

Proceed as described above in point 3.2.

3.7. Adding the mounting medium

Add 500 µl of mounting medium to each well.

3.8. Reading the results

Examine the plate under an inverted microscope equipped for fluorescence using an excitation filtre designed for fluorescein. If such equipment is not available the plate may be examined by means of a normal light microscope fitted for fluorescence, provided that the plate is turned upside down and low-power lenses are used. For higher magnification (40X), use lenses with long focal lengths. This will enable you to focus on the cells despite the thickness of the plastic.

3.9. Interpreting the results

If the specimen contains infectious haematopoietic necrosis virus, fluorescent green plaques will be visible in the cell layer. The higher the initial titre of virus in the inoculated material, the greater the number of these plaques. The sizes of these plaques will depend on the amount of time that the virus is allowed to replicate. These plaques may exist even in the absence of a cytopathogenic effect. The fluorescence must occur in the same focal plane as the cell layer. If "crystals" of fluorescence occur, the diluted solution may be passed through a 0.22 mm filtre to eliminate the aggregates. At low dilutions the specimen itself may be toxic to the cell layer. In this case or in any other situation leading to degeneration of the cell layer it may become