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From the State Veterinary Serum Laboratory, Department for Jutland, Århus, Denmark.

INDIRECT FLUORESCENT ANTIBODY TECHNIQUES FOR DEMONSTRATION OF TROUT VIRUSES AND CORRESPONDING ANTIBODY

By

P. E. Vestergård Jørgensen

JØRGENSEN, P. E. VESTERGÅRD: *Indirect fluorescent antibody techniques for demonstration of trout viruses and corresponding antibody.* Acta vet. scand. 1974, *15*, 198—205. — Two variations of the indirect fluorescent antibody technique (FAT) have been utilized in the work concerning two important virus diseases of trout, viral haemorrhagic septicaemia (VHS) and infectious pancreatic necrosis (IPN). A "two layer" indirect FAT allowed demonstration of the respective viruses in cell cultures and a "three layer" indirect FAT allowed demonstration of trout antibody to the viruses. Antibody, by means of the latter technique, could be demonstrated only in artificially immunized trout.

trout viruses; trout antibody; indirect fluorescent antibody techniques.

Direct fluorescent antibody techniques (FAT) for demonstration of the virus of viral haemorrhagic septicaemia (VHS) and infectious pancreatic necrosis (IPN) have previously been described (*Jørgensen & Meyling* 1972, *Grauballe* 1972). Staining of the two viruses by those techniques requires the use at low dilution of a fluoresceinisothiocyanate (FITC) labelled antiserum to each virus. In the present experiment an indirect FAT has been developed in which a FITC labelled swine antiserum to rabbit IgG will do for demonstration of both viruses, and which is more economic as regards viral antisera. Additionally, an indirect FAT has been developed which allows demonstration of antibody to the two viruses in trout serum.

MATERIAL AND METHODS

Virus infected cell cultures

Cultures of FHM (*Gravell & Malsberger* 1965) or RTG-2 cells (*Wolf & Quimby* 1962) on 22 by 9 mm cover glasses (18 such in a glass Petri dish with a diameter of 10 cm) were infected with dilutions of Egtved virus, strain No (serologically related to the reference strain F-1 (*Jensen* 1965)) or IPN virus, strain Sp or Ab (*Jørgensen* 1972 a). After infection the cultures were covered with a semisolid overlay medium containing 2 % methyl cellulose or 0.15 % agar (pH 7.6 to 7.8). After incubation at 15°C for 24 to 48 hrs. the overlay medium was removed by suction and the cell sheets rinsed three times in PBS (pH 7.4). Subsequently the cultures were fixed for 10 min. in acetone and stored at —20°C until used. The cell culture medium used throughout the experiment was Eagle's MEM with 10 % foetal bovine serum. During outgrowth the buffer was bicarbonate (CO_2-incubator), during plaque formation bicarbonate plus tris.

Rabbit antisera

Sera from rabbits immunized with Egtved or IPN virus as previously described (*Jørgensen* 1969, 1972 a) were used in dilution 1:100.

Antiserum to trout immunoglobulin was obtained by immunizing rabbits with purified immunoglobulin from trout inoculated with IPN virus (*Jørgensen* 1973). The working dilution of this antiserum was 1:64.

Trout sera

The following sera were examined for the presence of FA stainable antibody: 1, monthly individual serum samples from three trout immunized with Egtved virus (*Jørgensen* 1971); 2, serum pools from trout immunized with IPN virus (*Jørgensen* 1973); 3, five serum pools from trout exposed to natural infection with Egtved virus in five Danish trout farms where VHS is endemic. Neutralizing antibody to Egtved virus has not yet been demonstrable in such sera (*Jørgensen* 1971); 4, two pools of trout serum with high titres of neutralizing antibody to IPN virus, one originating from an IPN attacked trout farm, KiMs, the other from an "IPN-free" farm, RaMs (*Jørgensen* 1973).

The trout sera had been kept at —20°C for up to five years.

Conjugate

FITC labelled swine antiserum to rabbit IgG was obtained commercially (Dakopatts A/S, Copenhagen) and used at a 1:50 dilution.

Technique for virus demonstration ("two layer" indirect FAT)

Fixed cell cultures with virus plaques were covered with viral antiserum and incubated for half an hour in a moist chamber at 37°C. After rinsing in PBS (pH 7.4) for half an hour (three changes) the cultures were covered with conjugate and again incubated as above. Following another rinse as above the cultures were mounted in a mixture of glycerol and PBS (pH 7.4) (*Goldman* 1968) and examined by fluorescence microscopy as previously described (*Jørgensen* 1972 b).

Technique for demonstration of trout antibody ("three layer" indirect FAT)

Fixed cell cultures with virus plaques were treated as above (incubated and subsequently rinsed) with the following reagents in the order mentioned: 1, trout serum; 2, rabbit antiserum to trout immunoglobulin; 3, conjugate. Mounting and microscopy was carried out as described above.

Controls

Normal rabbit and trout serum served as specificity controls.

RESULTS

It was possible to demonstrate all of the three examined virus strains in cell cultures by means of the "two layer" indirect FAT (Table 1). Egtved virus was stained only after treatment with anti-Egtved serum; the two IPN virus strains, Sp and Ab, were stained only after treatment with an IPN antiserum. Antisera against strains Sp and Ab were both cross reactive since they induced staining also of the heterologous IPN virus strain, although at a lower level of fluorescence than with the homologous IPN virus strain.

FA stainable antibody could be demonstrated by the "three layer" indirect FAT in serum from trout artificially immunized with Egtved or IPN virus (Tables 2 and 3) but not in any of the other trout sera examined.

Table 1. Staining of Egtved and IPN virus in cell cultures by means of a "two layer" indirect fluorescent antibody technique.

Antigen	Antiserum	Conjugate	Fluorescence
Egtved virus, strain No (plaques in FHM cells)	rabbit anti- F-1, 1:100	FITC labelled swine antiserum to rabbit IgG. 1:50	+
—	normal rabbit serum, 1:100	—	0
—	rabbit anti-IPN(Sp), 1:100	—	0
—	rabbit anti-IPN(Ab), 1:100	—	0
IPN virus, strain Sp (plaques in FHM cells)	rabbit anti-IPN(Sp), 1:100	—	+
—	rabbit anti-IPN(Ab), 1:100	—	+
—	normal rabbit serum, 1:100	—	0
—	rabbit anti-Egtved(F-1), 1:100	—	0
IPN virus, strain Ab (plaques in RTG-2 cells)	rabbit anti-IPN(Ab), 1:100	—	+
—	rabbit anti-IPN(Sp), 1:100	—	+
—	normal rabbit serum, 1:100	—	0
—	rabbit anti-Egtved(F-1), 1:100	—	0

DISCUSSION

The semisolid overlay media used in this experiment proved to be effective plaquing media and yet were easily removable by suction and washing. The latter feature means that the cultures could be efficiently rinsed before fixation, unlike cultures covered with a solid overlay medium.

The use of cultures with virus plaques led to an easily observable contrast between the bright fluorescence from infected cells (areas with the characteristic shape of plaques) and the slight fluorescence from normal cells (between the plaques)

Table 2. Demonstration of trout antibody by means of a "three layer" indirect fluorescent antibody technique.

Antigen	Antibody	Antiserum	Conjugate	Fluorescence
Egtved virus (No) (plaques in FHM cells)	trout antiserum to Egtved virus (F-1), 1:2 to 1:96	rabbit antiserum to trout immunoglobulin, 1:64	FITC labelled swine antiserum to rabbit IgG, 1:50	+
—	normal trout serum, 1:1	—	—	0
—	trout antiserum to Egtved virus (F-1), 1:2	normal rabbit serum, 1:64	—	0
—	trout antiserum to IPN virus (Sp), 1:10	rabbit antiserum to trout immunoglobulin, 1:64	—	0
IPN virus (Sp) (plaques in FHM cells)	trout antiserum to IPN virus (Sp), 1:2 to 1:2560	—	—	+
—	normal trout serum, 1:1	—	—	0
—	trout antiserum to IPN virus (Sp), 1:2	normal rabbit serum, 1:64	—	0
—	trout antiserum to Egtved virus (F-1), 1:2	rabbit antiserum to trout immunoglobulin, 1:64	—	0
IPN virus (Ab) (plaques in RTG-2 cells)	trout antiserum to IPN virus (Sp), 1:10	—	—	+

caused by antibody to cell material or by non specific binding of antibody.

By direct FAT, IPN virus, strain Ab, could not be stained by heterologous antiserum conjugate (*Jørgensen* 1972 b), whereas in the present experiment both strains of IPN virus could be stained after treatment with heterologous antiserum. Besides, the background fluorescence was less pronounced in the indirect than in the direct FAT, probably due to the higher serum dilution used. The indirect FAT therefore would seem to be very well fitted for identification of IPN and Egtved virus.

Table 3. Demonstration of antibody in trout immunized with Egtved virus (repeated injections) or IPN virus (one injection).

	Time after first virus injection	Neutralizing antibody	Antibody stainable by means of indirect FAT
Egtved virus	1 month	0	0
	2 months	0	0
	3 —	0	0
	4 —	0	0
	5 —	0	0
	6 —	0	0
	7 —	0	+
	8 —	0	+
	9 —	0	+ (1:96)***
	10 —	0	+
	11 —	0	+
	12 —	+ (1:16)*	+
IPN virus	3 weeks	0	0
	6 —	+	+
	8 —	+ (1:4100)**	+ (1:2560)***
	12 —	+	+
	13 —	+	+

* 50 % end point titre in tubes.
** 50 % plaque-reduction titre.
*** highest serum dilution yielding fluorescence.

In one trout, FA stainable antibody to Egtved virus could be demonstrated five months earlier than neutralizing antibody, i.e., seven months after the first virus injection. The remaining two trout did not develop any of the two types of antibody (*Jørgensen* 1971). FA stainable antibody to IPN virus could be demonstrated in serum pools six weeks after the virus injection. At that time a rise in the titre of neutralizing antibody could also be demonstrated (*Jørgensen* 1973).

The "three layer" indirect FAT did not allow demonstration of antibody in trout exposed to natural infection with the viruses, not even in trout which contained neutralizing antibody to IPN virus at titres similar to those observed in the artificially immunized trout (1:3000 to 1:4000) (*Jørgensen* 1973). It thus appears that the chemical structure of neutralizing antibody in artificially immunized trout must differ in some respect from that of neu-

tralizing antibody in trout exposed to natural infection. The difference may be caused by the use of adjuvant in the immunization process, but is nevertheless surprising, since it has previously been found (*Jørgensen* 1973) that the two "types" of antibody appeared to belong to the same immunoglobulin class.

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SAMMENDRAG

Indirekte immunfluorescens-teknik til påvisning af ørredvirus og ørredantistof.

To ørredpatogene virus (Egtved og IPN virus) er påvist i inficerede cellekulturer ved hjælp af en „to-lags" indirekte immunfluorescensteknik. Ved hjælp af en „tre-lags" indirekte immunfluorescensteknik kunne antistof overfor de pågældende virus påvises tidligere end eller samtidig med neutraliserende antistof i serum fra kunstigt immuniserede ørreder. Metoden tillod ikke påvisning af antistof i ørreder udsat for naturlig smitte med Egtved eller IPN virus.

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Reprints may be requested from: P. E. Vestergård Jørgensen, State Veterinary Serum Laboratory, Dept. for Jutland, Hangøvej 2, DK-8200 Århus N, Denmark.