ISOLATION AND IDENTIFICATION OF INFECTIOUS PANCREATIC NECROSIS VIRUS FROM RAINBOW TROUT (*SALMO GAIRDNERI* RICHARDSON) IN SOUTH AFRICA.

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A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the Degree of Master of Science.

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ABSTRACT

ISOLATION AND IDENTIFICATION OF INFECTIOUS PANCREATIC NECROSIS VIRUS FROM RAINBOW TROUT (SALMO GAIRDNERI RICHARDSON) IN SOUTH AFRICA.

BRAGG, Robert Richard, University of the Witwatersrand.

A virus, serologically similar to the VR299 serotype of infectious pancreatic necrosis virus (IPN) was isolated from rainbow trout (Salmo gairdneri Richardson) in South Africa for the first time.

A total of 3 147 samples of rainbow trout, from 137 cases were submitted to the Fish Disease Unit of the Veterinary Research Institute. The virus was isolated from 11 of these cases. In addition to this, a total of 7 133 viscera samples and 2 892 ovarian fluid samples from asymptomatic fish was collected. A virus serologically similar to the VR299 serotype of IPN virus was isolated from viscera samples from three sites and from ovarian fluid samples from one site. A total of 5 200 eyed ova was also examined and a similar virus was isolated from one consignment of ova imported from the USA.

All the isolations were done on cell cultures and to this end the procedures for the passage and propagation of the RTG2, BF2, FHM and BB cell lines were established. It was found that the FHM cells inexplicably lost their susceptibility to the trout viruses. Thus the cell lines of choice for the isolation of viruses from trout in South Africa were the RTG2 and BF2 cell lines.

The viruses were serologically identified by the serum neutralization test, indirect fluorescent antibody technique and the newly developed direct immunostaphylococcus-protein-A (ISPA) test. The direct ISPA test makes use of the IgG binding characteristics of Protein A which is found in the cell membrane of Staphylococcus aureus Cowan strain A. The procedures for the direct ISPA test are similar to those for the direct fluorescent antibody technique.

All the viruses isolated in South Africa were serologically similar to the VR299 serotype of IPN virus which is a North American serotype. A similar virus was isolated from eyed trout ova imported from the USA and for these reasons it is proposed that the virus was introduced into South Africa via ova imported from the USA.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

R L Bragg

(Name of candidate)

22nd day of March, 1984.

CONTENTS

1. INTRODUCTION.

The rainbow trout (Salmo gairdneri Richardson, 1938) is a member of the salmonid family of fish which originated in the northern-temperate and subarctic areas (Needham, 1969; Sterba, 1967). This species now has an almost worldwide distribution as it has been introduced to all continents except Antarctica (Moyle & Cech, 1982).

The first attempts to introduce rainbow trout into South Africa were in 1896, but these were unsuccessful. In 1897 more rainbow trout were introduced and the fish which survived became the first brood stock at the Jonkershoek hatchery near Stellenbosch (McC Pott, 1987). The first rainbow trout to be introduced into Natal were obtained from the Jonkershoek hatchery in 1899 (Pike, 1980).

The first river to be stocked with trout was the Eerste river near Stellenbosch. This was done by the Stellenbosch Angling Club in 1899 (Hoy, 1916). Rivers in the Ceres area were stocked in 1901 (Hoy, 1916) and the Groot Drakenstein Angling Club stocked rivers in the Paarl and Franschoek areas with about 150 000 fry in 1905 (Hoy, 1916). Trout were later introduced into the Hex River and a number of other rivers in the area in 1915 by the Worcester Angling Club (Hoy, 1916).

In the Eastern Cape, the Frontier Acclimatization Society was established in 1894 with the aim of introducing trout into the area. An expanse of water at Amalinda near East London was opened to the public and stocked with trout. In 1915 the stocking of this water was stopped. In 1897 the Government opened a hatchery in the Perie Forests near King Williams Town (Hoy, 1916) which is still operational.

The Transvaal Acclimatization Society was established with the main aim of identifying suitable trout waters in the Transvaal. A hatchery was estabilised at Potchefstroom but this was unsuccessful due to high temperatures and a lack of water. The first records of successful introduction of trout into the Transvaal was in the Carolina area in 1914 (Hoy, 1916). Today most of the trout produced in the country come from the Eastern Transvaal. There were attempts to introduce trout into the Orange Free State but all of these early attempts were unsuccessful (Hoy, 1916).

The first Government trout hatchery was built at Newlands in 1892, but this soon proved to be too small and the hatchery at Jonkershoek was established in 1894 (McC Pott, 1987). Trout are

still produced on a small scale at the Jonkershoek hatchery in the Cape, although the Cape Department of Nature Conservation is no longer involved in the stocking of public waters with trout.

The Natal Parks Board also established a number of hatcheries. The first of these was established on the farm Tetworth on the Jackson river (Pike, 1980). In 1946 a hatchery was established at Underberg. Attempts were made to construct a hatchery at Giants Castle Game Reserve but these were unsuccessful. The hatchery at Royal Natal National Park was established in 1950 and the largest of the Natal Parks Board hatcheries, at Kamberg Nature Reserve, was built in 1975 (Pike, 1980). A hatchery was also established in the Transvaal at Lydenburg which was officially opened in 1955. This hatchery supplied trout ova to farmers in the Transvaal until recently. There is still a large brood population of rainbow trout at this hatchery.

The first trout farm in the country was established in 1945 by Mr R.R. Charter at Maloney's Eye near Johannesburg (McC Pott, 1979). In 1968 an outbreak of an undiagnosed disease caused extensive mortalities resulting in the closure of this farm (McC Pott, 1979).

Today, trout are produced in all four provinces of the country. A large portion of the table fish produced in South Africa comes from the Machadodorp, Lydenburg, Dullstroom, Waterval Boven and Sabie areas of the Eastern Transvaal. Some efforts have been made to produce trout in waters closer to Johannesburg but these enterprises have met with varying success.

Trout are also produced in the Western Cape by a few large enterprises. At present there are two farms in Natal which produce trout for the market. Both these farms are in the Drakensberg. There are a number of other farms in Natal but most of the production from these farms is used for the stocking of angling dams in the area. There are also two large farms in Qwa Qwa in the Orange Free State which produced about one tenth of the national annual production in 1985 (McC Pott, 1987).

Four basic methods for trout production are used in South Africa. The simplest method is in earth ponds which are dug in the ground. Examples of this method can be seen in Fig 1. The American style concrete raceway system is also used. This consists of a long narrow canal with a water inflow at one end and an outflow at the other end. Examples of the raceway system can be seen in Fig 2. The system most commonly used on South African trout farms is the circular pond system (Fig. 3), while cage cultures can be used if large expanses of water are available (Fig 4).

Fig 1. An earth dam for the cultivation of trout at the Fisantekraal trout hatchery in the Cape Province.

Fig 2. American style concrete race-way as seen at the Natal Parks Board hatchery at Kamberg.

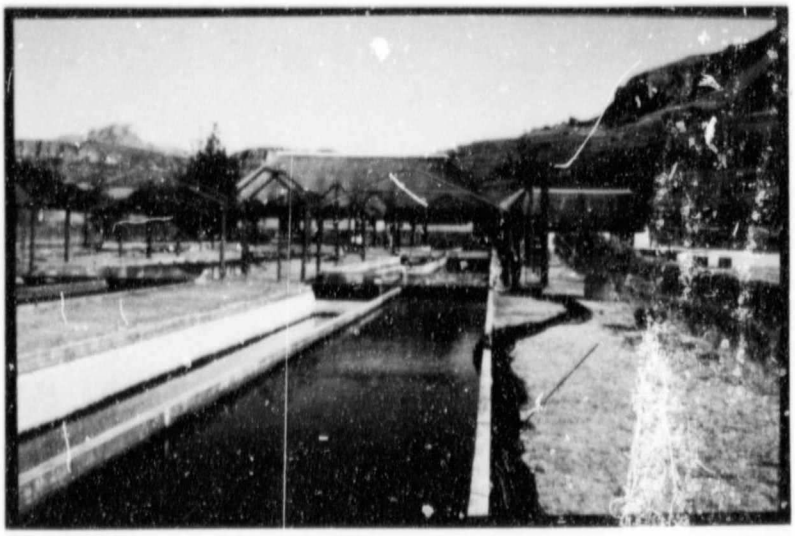


Fig 3. Circular trout production ponds as seen at the farm, 'Waterval' in the Lydenburg area of the Eastern Transvaal.

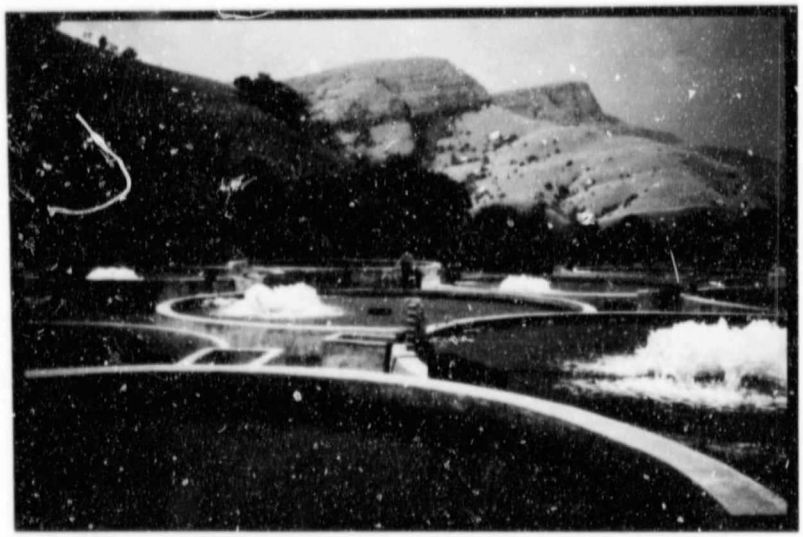
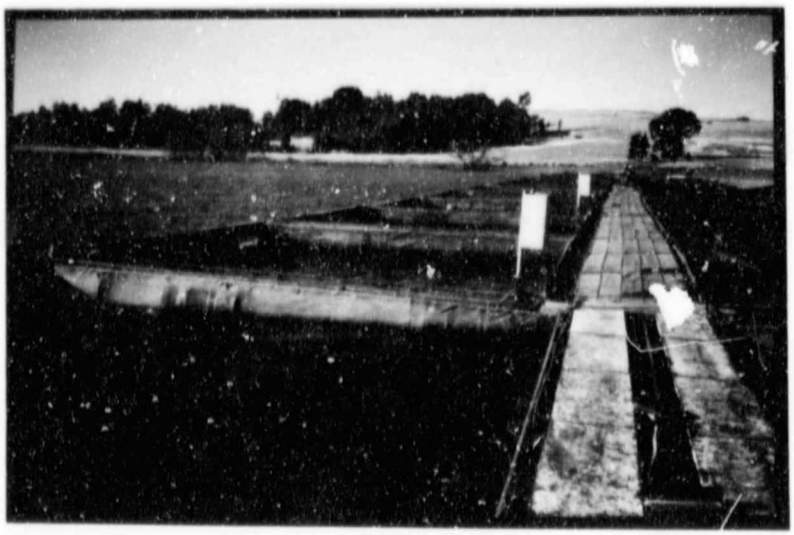


Fig 4. Cage culture system as seen at 'Aquacultura Pty Ltd' in the Belfast area of the Eastern Transvaal.



Trout production in South Africa totalled about 461 tons during 1985 with an estimation of about 520 tons for 1986 (McC Pott, 1987). The market for table trout in South Africa is limited however, due to high prices resulting from high production costs. This has forced some of the producers to look for alternative markets, such as the export of trout ova to the Northern Hemisphere. Trout only breed during the winter and for a constant production of market size fish, ova must be obtained during the summer. At present the only feasible source of 'summer ova' is the Southern Hemisphere where only a few South American countries, New Zealand, Australia and South Africa are capable of supplying ova to the Northern Hemisphere.

The only major problem restricting the export of ova to Europe is the very stringent health requirements of most of the countries. Most countries insist on a health certificate which states that the fish from which the ova were obtained had been tested for the presence of viruses and found to be free for the previous two years at least.

The great international demand for South African trout ova has resulted in a need for the virological examination of the trout in this country. Before this could be undertaken, the methods for the isolation and identification of the viruses had to be established and verified. Although the test procedures required for the health certification of the fish are standard and well documented, these procedures had not been done in South Africa before. To this end, samples of fish cell lines were obtained and passaged. Samples of the different serotypes of infectious pancreatic necrosis virus (IPN virus), infectious haematopoietic necrosis virus (IHN virus), viral haemorrhagic septicaemia virus (VHS virus) and herpesvirus salmonis were obtained and the cytopathic effects (CPE) of these viruses on the different cell lines were studied. The procedures for the serological identification of IPN virus were also investigated in detail. The procedures for the serological identification of VHS virus and IHN virus are similar to those for IPN virus and were thus not investigated in the same detail.

The final and most important part of this project was to collect samples of diseased trout, viscera and ovarian fluid of asymptomatic fish and trout ova to perform the isolation procedures on these samples to determine the virological status of the rainbow trout in South Africa.

2. LITERATURE REVIEW

The study of fish viruses is a relatively new field of virology, with most of the current knowledge being acquired since 1951 (Pilcher & Fryer, 1980; Wolf, 1982). The main reason for the increasing interest in the viral diseases of fish is a result of the world wide increase in the cultivation of fish for food as well as for sport. Most of the information on viral diseases of fish comes from work carried out on members of the salmonid family (Wolf, 1966). This is due to the fact that the salmonids are the most commonly cultivated species of fish. The first successful isolation of a virus from a fish was in 1957 (Wolf, Quimby, Pyle & Dexter, 1960) when infectious pancreatic necrosis virus was isolated from trout. Since this first isolation, viruses have been isolated from a wide range of fish, and a viral etiology has been established or suspected in more than 50 diseases (Wolf, 1988). There has been a very rapid increase in knowledge on viral diseases of fish. This is illustrated by the fact that in 1982 Wolf only listed 17 diseases for which a viral etiology was proven and a further 15 diseases for which a viral etiology was suspected (Wolf, 1982), yet in 1988 this number had increased to 50 (Wolf, 1988). The list of viral diseases of fish is ever increasing as more research is being undertaken and other species of fish are subjected to intensive culturing.

Fish viruses have characteristics similar to some groups of viruses from other host groups and some of the fish viruses have been included into the rhabdovirus, herpesvirus, birnavirus, and adenovirus groups. It has been found that both DNA and RNA viruses and even double stranded RNA viruses are represented among the fish viruses. There is, however, one major difference between viruses isolated from fish and other animals. This is the optimal temperature for replication. The optimal incubation temperatures for viruses from salmonid fish are between 10-20C, while the temperature for viruses from warm-water fish has been found to be between 20-27C. The optimal incubation temperature for viruses from homeothermic vertebrates has been found to be between 33-38C.

Of the 50 viral diseases, or suspected viral diseases, of fish Wolf, 1988), only five have been found to affect rainbow trout. These are infections with infectious pancreatic necrosis virus (IPN virus), infectious haematopoietic necrosis virus (IHN virus), viral haemorrhagic septicaemia virus (VHS virus), herpesvirus salmonis, and intraerythrocytic virus of rainbow trout.

2.1. Infectious pancreatic necrosis virus (IPN virus).

The first report of a disease resembling IPN was made in 1941 by M'Gonigle from Canada who reported an outbreak of a disease in brook trout. This disease was termed 'acute catarrhal enteritis' (M'Gonigle, 1941). Wood, Snieszko & Yasutake (1955) investigated a similar disease in brook trout hatcheries in 1953 and suggested a viral etiology. From the histopathology they named the disease infectious pancreatic necrosis (Wood et al, 1955). The infectious nature of the disease was determined (Snieszko, Wolf, Camper & Pettijohn, 1959) and the virus was eventually isolated in 1960 (Wolf et al, 1960). IPN virus has been classified as a member of the birnavirus group due to the double stranded bi-segmented RNA of the viral genome (Dobos, Hill, Hallet, Kells, Becht & Teninges, 1979). Since the first isolation of IPN virus, this virus has been found to have an almost world wide distribution. The virus has been isolated from fish in the USA (Wolf et al, 1960; Parisot, Yasutake, & Bressler, 1963), Canada (MacKelvie & Artsob, 1969), Europe (Besse & de Kinkelin, 1965; Vestergard-Jorgensen & Bregnballe, 1969; Ball, Munro, Ellis, Elson, Hodgkiss & McFarlane, 1971; Schlotfeldt, Liesse & Frost, 1975), Japan (Sano, 1971), Chile (McAllister & Reyes, 1984), Korea (Hedrick, Eaton, Fryer, Hah, Park, & Hong, 1985), Taiwan (Hedrick, Fryer, Chen & Kou, 1983) and South Africa (Bragg & Combrink, 1987a). IPN virus has not been isolated from fish in Australia (Langdon, Humphrey, Copland, Carolane, Gudkovs & Lancaster, 1986).

IPN virus and other birnaviruses morphologically and serologically similar to IPN virus have been isolated from a number of different species of fish, both salmonid and non-salmonid (Adair & Ferguson, 1981; Hudson, Bucke & Forrest, 1981; Schutz, May, Kraeuter & Hetrick, 1984). McAllister (1983) listed a total of 15 species of salmonid fish and 14 species of non-salmonid fish from which IPN or 'IPN-like' viruses have been isolated. IPN virus has also been isolated from marine and estuarine fish (McAllister, Newman, Sauber & Owens, 1983; 1984; Bonami, Cousserans, Weppe & Hill, 1983). Clinical signs of disease, however, have only been noted in brook trout (M'Gonigle, 1941), rainbow trout (Hill, 1982), brown trout and atlantic salmon (MacKelvie & Artsob, 1969). IPN or 'IPN-like' viruses have been isolated from marine shellfish (Hill, 1976b; Underwood, Smale, Brown & Hill, 1977) but the pathogenicity of these isolates for shellfish is still unconfirmed (Hill, 1982).

The clinical form of IPN virus infection appears to be limited to fish kept under intensive hatchery conditions (Munro, Liversidge & Elson, 1976) and epizootics have not been reported from wild fish (Hill, 1982). The first signs of an infection with IPN virus include a sudden increase in mortality amongst the largest, fastest growing fry or fingerlings (Pilcher & Fryer, 1980; Hill, 1982; Roberts & Shepherd, 1986). The fish are usually dark in colour, have exophthalmia, distended abdomen and often trail mucoid pseudocasts from the vent. The fish swim in an erratic manner with a corkscrew motion. Unfortunately fish with a number of bacterial as well as other viral infections exhibit similar symptoms and none of these symptoms are very characteristic for IPN virus (Wolf, 1966). Internal examination reveals a pale liver and spleen, frequently together with large amounts of whitish mucus in the lumen of the stomach and intestine. Petechial haemorrhages can often be observed in the pyloric caeca and pancreatic tissue. The histopathology of IPN includes severe necrosis of the pancreatic acinar cells, with nuclear piknosis, karyorrhexis and occasional intracytoplasmic inclusion bodies. (McKnight & Roberts, 1976).

It has been found that 'first feeding' fry are the most susceptible to the disease, with an increasing resistance with increasing age to about 5-6 months (Hill, 1982; McAllister & Reyes, 1984). There has, however, been a report of IPN in yearling rainbow trout (Elazhary, Lagace, Cousineau, Roy, Berthiaume, Paulhus & Frechette, 1976). Roberts & McKnight (1976) also reported a stress-mediated re-occurence of the disease in fish which had survived an outbreak of IPN.

IPN virus is the most thoroughly characterized of all the fish viruses (Hill, 1982) with detailed reports on the biochemical (Kelly & Loh, 1972; Underwood et al, 1977; Chang, MacDonald & Yamamoto, 1978), biophysical (Dobos, Hallett, Kells, Sorensen & Rowe, 1977) and physico-chemical (Cohen, Poinsard & Scherrer, 1973) characteristics of the virion and its genetic material (Dobos 1976, MacDonald & Yamamoto, 1977). From electron microscope studies done on the virus (Moss & Gravell, 1969; Kelly & Loh, 1972; Hill, 1982) it was found that the virus is icosahedral with a diameter of 59 - 71 nm. Each capsid is composed of 180 structural sub-units shared by 92 pentagonal and hexagonal capsomeres. It was determined that the IPN virus has four polypeptides of approximate molecular weights of 90,000, 60,000, 29,000 and 27,000 daltons by polyacrylamide-gel electrophoresis (Dobos et al, 1977). It was found that the genome of IPN virus makes up 8.7% of the total viral mass (Hill, 1982). The

genetic material consists of two double stranded RNA segments (Dobos, 1976), with an approximate molecular weight of between 2.2×10^6 to 2.3×10^6 for the one segment and 2.4×10^6 to 2.5×10^6 for the other segment (Dodos, 1976; Hill, 1982).

The serology of IPN virus is very complex. Cross-neutralization studies (Wolf & Quimby, 1971; Vestergard-Jorgensen & Kehlet, 1971; Lientz & Springer, 1973) show that there is quite a wide antigenic diversity amongst the strains of IPN virus. Hill (1982) identified four serotypes of IPN virus in Europe which differ markedly from the three or four serotypes of the virus isolated in the USA and three strains isolated in Canada (MacDonald, Moore & Souter, 1983). The recent development of monoclonal antibodies against the West Buxton (North American) serotype of IPN virus (Caswell-Reno, Reno, & Nicholson, 1986) may assist in the clarification of the serological differences between the different isolates of IPN virus. There are three serotypes of IPN virus which are most commonly used as reference serotypes. These are the VR299 serotype which was isolated from fish in the USA and the Ab and Sp serotypes which were both isolated from fish in Europe.

2.2. Infectious haematopoietic necrosis virus (IHN)

The first report of a disease which could have been infectious haematopoietic necrosis was made by Rucker, Whipple, Parvin & Evans (1953) who investigated a disease in sockeye salmon. Unfortunately, the virus was lost during storage. Another outbreak of a disease in juvenile sockeye salmon was investigated and a virus was isolated from these fish (Wingfield, Fryer & Pilcher, 1969). Wingfield *et al* (1969) described the virus and it was proposed that this was the same virus which had been isolated by Rucker *et al* (1953). The first report of a similar infection in rainbow trout was in 1967 (Amend, Yasutake & Mead, 1969). The histopathology of this infection revealed necrosis of the haemato poietic tissue of the kidney and spleen and the disease was named 'Infectious haematopoietic necrosis' (Amend *et al*, 1969).

The virus is bullet shaped (Amend & Chambers, 1970) with an average length of 160nm and an average width of 90nm. It has an outer coat of 15nm thick and an inner core of 60nm in diameter (Amend & Chambers, 1970). The nucleic acid of IHN virus consists of RNA (McCain, Fryer & Pilcher, 1974). From the results

obtained from the ribonuclease sensitivity and the non-equimolar ratio of adenylic and uridylic acids it can be seen that the RNA is single stranded (Pilcher & Fryer, 1980). In the light of the above information IHN virus was classified as a rhabdovirus (Amend, 1974).

IHN virus has been isolated in the USA from sockeye salmon, chinook salmon, rainbow trout (Amend, 1974) steelhead trout and cutthroat trout (Mulcahy, 1984). Sano, Nishimura, Okamoto Yamazaki, Hanada & Watanabe (1977) reported the isolation of IHN virus from rainbow trout in Japan, and it has recently been reported in Europe (Bovo, Giorgetti, Vestergard-Jorgensen & Olesen, 1987). The virus has not been reported in fish from Australia (Langdon et al, 1986) or Africa.

Fish infected with IHN virus become lethargic, with sporadic whirling and hyperactivity (Amend, 1974). There is usually a sudden increase in the mortality rate and the fish have a dark colour (Pilcher & Fryer, 1980). There is often exophthalmia, an abdomen distended with fluid and faecal casts (Pilcher & Fryer, 1980; Roberts & Shepherd, 1986). The spleen, kidney and liver are pale while the stomach is filled with a milky fluid. There are petechial haemorrhages in the visceral adipose tissue. Microscopically, the most outstanding feature is necrosis of the haematopoietic tissue of the kidney and spleen. The environmental temperature appears to be very important in IHN virus infection and it has been found that epizootics do not occur if the water temperature is above 15C. Fish of all ages appear to be affected by IHN virus (Pilcher & Fryer, 1980).

2.3. Viral haemorrhagic septicaemia virus (VHS).

The first report of VHS was from diseased rainbow trout near a small village called Egtved in Denmark (Jensen, 1963). The disease was first called 'Egtved disease'. A viral etiology for this disease was established after the isolation of a virus by Jensen (1965). The virus was described as being morphologically similar to a rhabdovirus after electron microscope investigations (Zwillenberg, Jensen & Zwillenberg, 1965). These results were confirmed by de Kinkelin & Scherrer (1970). The length of the virus was found to be between 100-200 nm (Zwillenberg et al, 1965) with a diameter of 70-80 nm. Scherrer (1973) found a length of between 180-240 nm with a diameter of 60 - 75 nm. Hill,

Underwood, Smale & Brown (1975) found that the nucleic acid from VHS virus was RNA and the single stranded nature was established by Robin & Rodrigue (1977). From these results VHS virus has been classified as a rhabdovirus.

This virus has only been isolated from fish in European and Scandinavian countries were it is a serious problem, resulting in the loss of about 20 000 to 30 000 tons of fish per year (de Kinkelin, 1982). The disease has been reported in Denmark, Czechoslovakia, France, Germany, Italy, Norway, Poland and Sweden (Wolf, 1972). The virus has not been isolated from the USA, Canada and Australia (Langdon et al, 1986).

The most susceptible species of fish is the rainbow trout, with the fingerlings being more susceptible than fry-fry and brood fish (Pilcher & Fryer, 1980). VHS virus has also been isolated from marine reared rainbow trout (Horlyck, Mellergard, Dalsgaard & Vestergard-Jorgensen, 1984). Experimental infections have revealed that sea bass, turbot (Castric & de Kinkelin, 1984) and atlantic salmon (de Kinkelin & Castric, 1982) are susceptible to VHS virus. The virus has also been isolated from diseased pike (Meier & Pfister, 1981) and from asymptomatic wild white fish (Ahne & Thomsen, 1985). It has been found that VHS normally only occurs when the water temperature is lower than 8C (Pilcher & Fryer, 1980). Horlyck et al, (1984) have reported VHS in rainbow trout reared in seawater at 11C to 14C and Roberts (1980) states that the upper temperature limit for VHS is 15C.

There are three stages in VHS virus infection. In the acute stage, mortalities increase suddenly and the fish have a dark colour. There is often exophthalmia, and haemorrhagic streaks in the gills and the connective tissue of the eye. In the chronic stage, the mortality rate drops, but mortalities persist for a long time. The fish become very dark in colour, and have severe exophthalmia. In the terminal stages of the infection, the fish swim in a spiral fashion with few other symptoms. Usually all three stages of the disease are present in a population (Pilcher & Fryer, 1980). The most characteristic pathological change is the scattered haemorrhages in the connective tissue, perivascular adipose tissue, swim bladder and intestine. The liver is often bright red in the acute stages but pale during the chronic stage. Microscopically, necrotic foci and cytoplasmic vacuolization of the hepatocytes can be seen. The kidney is reddened and thickened in the acute stage, but changes to a grey colour during the chronic stages. The histopathological examination of infected kidneys reveals necrosis in the nephrons, cytoplasmic vacuolization, detachment of the epithelium of the uriniferous tubules and glomerular oedema (Pilcher & Fryer, 1980).

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There was a report of 'VHS' in South Africa (Lombard, 1968) but there was no indication of any attempts to isolate the agent, or serologically identify it. The description of 'VHS' in South Africa was based on symptoms, which are not specific. It was also found that the addition of 0.003% Terramycin (Oxytetracycline) and a vitamin E preparation resulted in the immediate recovery of the fish. This is an indication that the disease was not VHS, as reported, but rather a bacterial infection.

2.4. Herpesvirus salmonis.

A virus was isolated from diseased post-spawning rainbow trout brood stock from a hatchery in Washington State (Wolf & Taylor, 1975). The virus characteristics were investigated by Wolf, Darlington, Taylor, Quimby & Nagabayashi (1978) who found hexagonal nucleocapsids with a diameter of about 90 nm in virus infected cells. They also found that the envelopment of the virus occurs at the nuclear membrane. (Wolf et al, 1978) called the virus herpesvirus salmonis. A herpesvirus was also isolated from Oncorhynchus nerka fry in Japan in 1970 (Wolf, Nagabayashi & Quimby, 1975a). Wolf, Sano & Kimura (1975b) stated that the virus isolated from diseased fish in Japan was the same as the virus isolated from the USA. Sano (1976) however, stated that the herpesvirus from fish in Japan was different to the one isolated in the USA. It would appear that herpesvirus salmonis affects rainbow trout and Atlantic salmon.

Affected fish become lethargic and lose motor control (Wolf et al, 1975b). External symptoms of a herpesvirus salmonis infection include a darkening of the skin, severe exophthalmia often with haemorrhages in the orbit of the eye and abdominal distension (Wolf et al, 1975b). Internally, there are large amounts of ascitic fluid, the liver, spleen and digestive tract are flaccid and the vascular organs are mottled with areas of hyperaemia (Wolf et al, 1975b). The histopathology of an infection with herpesvirus salmonis includes an increase in the haematopoietic tissue, while the kidney tubules are filled with serous material and the renal tissue is oedematous (Wolf et al, 1975b). The liver is possibly the target organ and appears oedematous with fatty infiltration and vascular stasis.

2.5. Cell culture

The advent of fish cell cultures was probably the most important single event which triggered off the rapid assimilation of knowledge on fish viruses (Agius, 1982).

Wolf & Mann (1980) listed a total of 61 fish cell lines from 36 different species of fish representing 17 different families. An additional 16 cell lines, including 10 lines from salmonid fish (Lannan, Winton & Fryer, 1984; Braun-Nesje, Kaplan & Seljelid, 1982), 2 lines from the atlantic sturgeon (Li, Marrayatt, Annand & Odense, 1985), 2 lines from tilapia (Chen, Ueno, Wen & Kou, 1983; Lewis & Marks, 1985) and 2 lines from other fish (Bowser & Plumb, 1980; Ribeiro, Ahne & Lichtenberg, 1983) can be added to this list. A total of 18 of these cell lines are from rainbow trout (Wolf & Mann, 1980; Lannan et al, 1984; Braun-Nesje et al, 1982).

The first cell line to be established from fish was from the gonads of rainbow trout and these cells were called the RTG2 cell line (Wolf & Quimby, 1962). This cell line is still very widely used and has been well characterized. The RTG2 cell line is one of the certified cell lines which are stored with the American Type Culture Collection (ATCC).

The procedures for the passaging of fish cell lines have been reviewed (Wolf & Quimby, 1973c; 1978; Hill, 1976a) and found to be similar to those used for homeothermic cell lines (Wolf & Mann, 1980). The only difference between the cells from homeotherms and poikilotherms is the optimal temperatures for growth, which is about 10 - 15C lower in the case of homeotherms.

The different fish cell lines have been extensively used for the study of viruses from fish and the procedures for the inoculation of these cells have been reviewed (Hill 1976a). It was found that the different trout viruses usually produce different and often characteristic cytopathic effects (CPE) on the cell sheet (Hill, 1976a). The CPE produced by IPN virus on RTG2 cells is very characteristic and normally starts with areas of feathery or stringy cells, with the cells retaining an elongated shape. The end result of the inoculation of a cell sheet with samples of IPN virus, is the complete destruction of the cells (Hill, 1976a; Roberts, 1980). The inoculation of the cells with samples of IHN virus resulted in the rounding off of the cells and the formation of clumps of cells (Roberts, 1980). VHS virus also causes a rounding off of the cells, with complete destruction of the cell sheet after a few days (Roberts, 1980).

2.6. Virus isolation procedures

There are numerous publications outlining the procedures for the isolation of a virus from a population of fish showing signs of a disease. Most of these procedures are very similar with only a few differences and these procedures have been reviewed (Wolf, 1970; Hill, 1976a; Amos, 1985). As far as possible, moribund fish should be used. Under no circumstances can frozen fish be used as many of the viruses are sensitive to freezing and thawing (Hill, 1976a). In the case of adult fish, samples of the liver, spleen, pyloric caeca and kidney should be removed for viral examination. In the case of fry, the fish can be processed whole (Wolf, 1970; Hill, 1976a, & Amos, 1985). The first stage in the processing of samples involves the disruption of the tissue. This can be carried out by grinding the cells in a mortar and pestle (McDaniel, 1979) or in a laboratory homogenizer (Hill, 1976a). Sonication has been found to be unsuitable for the disruption of tissue (Amos, 1985). The homogenates are diluted to a final dilution of 1/10 (Hill, 1976a) and bacterial contamination is removed. There are two approaches to the removal of bacterial contamination. The first and most widely used is filtration through 0,45µm filters (Wolf, 1970; McDaniel, 1979) and the other involves the incubation of samples with antibiotics (Amend & Pirtsch, 1972).

The processed samples are then inoculated onto susceptible cell lines by firstly removing the culture medium, followed by the addition of small volumes of virus containing medium. The cells are then incubated to allow for the adsorption of the virus (Adair & Ferguson, 1981). The virus containing fluid is removed and the fresh medium is added to the culture. The cells are then incubated at a temperature which will allow for the replication of the viruses for 7 days (Hill, 1976a) or 14 days (McDaniel, 1979; Amos, 1985)

Another source of viruses from trout is viscera from asymptomatic carrier fish, which had survived a viral infection (Billi & Wolf, 1969). There are two methods for the isolation of viruses from the viscera of asymptomatic fish. The first, and method of choice, is the same as for the isolation of a virus from diseased fish (Wolf, 1970; Hill, 1976a). A co-cultivation method for the isolation of viruses from viscera of asymptomatic fish was established by Agius, Mangunwiryo, Johnson & Smail (1982). Suspect tissue is subjected to trypsinization and the samples are inoculated onto RTG2 cells (Agius, et al). They claim that this technique is more sensitive than the conventional methods described above. This was confirmed by Agius, Richardson & Walker (1983), who compared five different procedures and found the co-cultivation technique to be the most sensitive. This technique has not, however been widely used by other authors who

prefer the more conventional methods. Agius, et al (1982) incubated the samples in antibiotics before trypsinization, after which no antibiotics was added. The technique would thus have to be carried out under strict aseptic techniques which could result in the loss of many samples from bacterial or fungal contamination.

Viruses can be isolated from ovarian fluid of carrier fish (Wolf, Quimby & Bradford, 1963) and this has been found to be the best source of IHN virus (Amend & Wedemeyer, 1970; Amos, 1985). The methods for the isolation of viruses from ovarian fluid have been reviewed by Hill (1976) and more recently by Amos (1985).

One of the most important aspects when attempting to isolate virus from asymptomatic populations of fish is to collect a statistically representative sample. The numbers of fish needed to make up a statistically representative sample have been discussed by Amos (1985) and Simon & Schill (1984). To detect one carrier fish if 2% of the population are carriers with 95% confidence 150 fish should be collected if the population is larger than 100 000 fish as is the case on most trout farms. Smaller samples are taken if the population is less than 100 000. Simon & Schill (1984) presented tables with exact sample sizes for various populations, confidence levels and disease incidences.

There is evidence to suggest that trout viruses are vertically transmitted to the offspring via the ova (Bullock, Rucker, Amend, Wolf & Stuckey, 1976; Mulcahy, Jenes & Pascho, 1984). Viruses have been isolated from trout ova by the methods described by Fijan & Giorgetti (1978).

Samples of diseased fish, ova, and statistically representative samples of ovarian fluid and viscera from asymptomatic fish should be collected during any attempts to isolate any of the trout viruses from fish, as these are the samples which are most likely to harbour the viruses of trout.

2.7. Serological identification of trout viruses.

Once a virus has been isolated from a diseased fish, viscera or ovarian fluid of asymptomatic fish or from eyed ova, the virus has to be identified. The viruses can be identified serologically if antisera against the viruses are available. The procedures for the preparation of viral specific antibodies in rabbits have

been reviewed by Hill, Williams & Finlay (1981). They found that high titres of neutralizing antibodies against IPN virus could be produced in rabbits. They also found that antisera with high titres against IHN virus and VHS virus were more difficult to produce.

A host of serological techniques for the identification of the different serotypes of IPN virus, VHS virus and IHN virus has been established. These serological tests include the serum neutralization test (Hill, 1976a; Lientz & Springer, 1973), the indirect immunofluorescent antibody test (Hill, 1976a; Piper, Nicholson & Dunn, 1973; Tu, Spendlove & Goede, 1974; Swanson & Gillespie, 1981; Vestergard-Jorgensen & Meyling, 1972), the indirect immunoperoxidase test (Nicholson & Henchal, 1978), the enzyme-linked immunosorbent assay (Nicholson & Caswell, 1982; Dixon & Hill, 1983), the complement fixation test (Hill, 1976a; Finlay & Hill, 1975), the viral induced bacterial co-agglutination test (Kimura, Yoshimizu & Yasuda, 1984) and the immunoblot assay (McAllister & Schill, 1986). The tests most often used for the serological identification of trout viruses are the serum neutralization test and the indirect immunofluorescent antibody test (Hill et al, 1981). The serum neutralization test, indirect immunofluorescent antibody test, indirect immunoperoxidase assay and the viral induced bacterial co-agglutination test were used to identify IPN virus during this study.

2.8. Detection of antibodies in the serum from fish

Another method to ascertain if any of the trout viruses are present in the country, is to test for the presence of virus specific antibodies in the serum of trout. Samples of blood can be collected from trout by cardiac puncture, puncture of the caudal artery or vein (Itazawa, 1957), puncture of the dorsal aorta (Schiffman, 1959) and puncture of the duct of Cuvier (Lied, Gjerde & Braekkan, 1975). The serum can be removed from the blood by allowing the blood to coagulate, and drawing off the serum. The presence of neutralizing antibodies in the serum of the fish can be detected by virus neutralization by either the plaque reduction method (Vestergard-Jorgensen, 1973) or by the determination of neutralizing indices (Wolf & Quimby, 1969).

Extensive work on the ability of trout, exposed to IPN virus, to produce antibodies has been undertaken. Wolf & Quimby (1969)

demonstrated that rainbow and brook trout could produce significant amounts of neutralizing antibodies against the VR 299 serotype if injected with the virus. They found that wild fish from a river with a hatchery where IPN had occured, had neutralizing antibodies against the virus, while serum from fish with no known contact with the virus had no neutralizing antibodies (Wolf & Quimby, 1969). Dorson & de Kinkelin (1974) undertook a survey of hatchery and wild fish in France and found neutralizing activity in the serum of hatchery fish, but not from wild brown trout. They found neutralizing activity in the serum of rainbow trout from IPN free farms (Dorson & de Kinkelin, 1974). Vestergard-Jorgensen (1973) found antibodies against the Sp serotype of IPN virus in all fish serum which he examined. In the light of the results obtained by both Vestergard-Jorgensen (1973) and Dorson & de Kinkelin (1974), care must be taken when interpreting results of any serological survey as both found significant levels of neutralizing antibodies in fish with no know history of exposure to IPN virus and the immunogenic stimulus for the immune response is unknown. The possible detection of neutralizing antibodies in the serum of fish in South Africa need not necessarily indicate past exposure to a virus.

3. METHODS AND MATERIALS

3.1. Tissue culture

The plastic disposable culture flasks used for the cultivation of fish cells were supplied by Sterilab Services. Two different types of culture flasks were used. The first type of flask had a volume of 200 ml and the surface on which the monolayer of cells could form was 75 cm^2. These flasks are referred to as 75 cm^2 flasks throughout. The other type of flask used had a volume of 50 ml and a surface of 25 cm^2. These flasks are referred to as 25 cm^2 flasks in this text.

Cell lines used for the isolation and identification of trout viruses were the rainbow trout gonad cells (RTG2) (Wolf & Quimby, 1962) ATCC No. CCL 55, bluegill fry cells (BF2) (Wolf, Gravell & Malsberger 1964) ATCC No CCL 91, brown bullhead cells (BB) (Cerini & Malsberger, 1962) ATCC No CCL 59 and the fathead minnow cells (FHM) (Gravell & Malsberger, 1965) ATCC No CCL 42. Samples of these cell lines were obtained from the American Type Culture Collection (ATCC) by Flow Laboratories who reconstituted the cells and supplied them in 75 cm^2 tissue culture flasks. Samples were also obtained from the ATCC by Sterilab Services, and these cells were supplied in a frozen state. The cells were reconstituted by placing them in a waterbath at 37C for 30 seconds to thaw. The cells were centrifuged at 125 x g for 10 minutes. The supernatant was discarded and the pellet resuspended in 10 ml Eagles Minimum Essential Medium (MEM)(Appendix 1) supplemented with 0.1ml of a 100x concentration of non-essential amino acids (NEAA)(Flow Laboratories) (Appendix 1), 1ml foetal calf serum (FCS) (Sterilab Services), 120 mg penicillin/litre and 200 mg streptomycin/litre. The cell suspension was placed into a 25 cm^2 culture flask and incubated at 22C in the case of the RTG2 cells and 25C in the case of the BF2, BB and FHM cells. The cells were observed daily and the growth recorded. All the cell lines were passaged once they were confluent.

The techniques used for the passage of fish cell lines were similar to those described by Wolf & Quimby (1973c; 1978) and Hill (1976a). The old culture medium was removed and discarded. The cultures were washed once with activated trypsin versin (ATV). Another sample of ATV was added to the cells which were incubated at 25C for 30 seconds. The ATV was discarded and the

cells were incubated at 25C until the cell sheet was seen to detach from the culture vessel. Fresh growth medium which contained 1% v/v NEAA, 10% v/v FCS and antibiotics, was then added to the cells. Any cells still attached were loosened by drawing the medium into a syringe through a 14 gauge needle. The cell suspension was transferred to an Erlenmeyer flask which contained fresh growth medium. The volume of the medium in the Erlenmeyer flask was dependent on the size of the culture flask which was passaged. In the case of a 25 cm^2 culture flask, the flask would contain 40 ml medium. In the case of 75 cm^2 culture flask, the Erlenmeyer flask would contain 120 ml medium. Smaller samples of the cell suspension were removed from the Erlenmeyer flask and new cultures were seeded. In the case of 25 cm^2 culture flasks, 10ml of the cell suspension was used, while for the 75 cm culture flasks, 30 ml of the suspension was used. When 24 well plates were used, each well was filled with 1 ml of the cell suspension.

When passaging the cells, the cultures were divided into three new cultures, i.e. one 75 cm^2 culture flask would be split into three 75 cm^2 culture flasks. Stock cultures of cells were grown up in 75 cm^2 flasks, and these cultures were used to seed the plates which were used for the isolation and identification of the trout viruses. One 75 cm^2 culture flask can be divided into either three 24 well plates, three 12 well plates or 30 petri dishes depending on the tests to be carried out. One 25 cm^2 culture flask could be used to seed either three 25 cm^2 culture flasks, one 75 cm^2 flask or one 24 well plate. All of the cultures were observed daily and the time needed for the cells to reach 100 % confluency was recorded.

Samples of the different cell lines were stored in liquid nitrogen. Evans, DeOca, Bryant, Schilling & Shannon (1962) outlined the procedures for the freezing of vertebrate cell lines and these techniques were used for freezing the fish cell lines (Wolf & Quimby, 1978). Cultures of the RTG2, BF2, FHM and BB cells were grown up in 75 cm^2 culture flasks. The cells were processed in the same way as for passages. After the cell sheet had been disrupted, 2 ml medium which contained 20% v/v FCS was added to the cells. The cell suspension was transferred to an Erlenmeyer flask which was kept on ice, and 2 ml medium containing 20% v/v dimethyl sulfoxide (DMSO) was added to the cells, one drop at a time. The DMSO was added to the cells to prevent the formation of water crystals in the cells during the freezing process (Perry, Kroener & Martin, 1975). The suspension was placed into cryo-preservation tubes and frozen slowly in a polystyrine container in the gas phase of the liquid nitrogen container. The cells were reconstituted by the same methods described above.

3.2. Virus inoculation procedures.

Before any attempts to isolate viruses from fish could be made, the effects of the different serotypes of IPN virus, IHN virus, VHS virus and herpesvirus salmonis on the different cell lines were studied. Samples of the VR299, Sp and Ab serotypes of IPN virus as well as a sample of VHS virus were supplied by Dr Barry Hill of the Fish Disease Laboratory, England. Dr Vestergard-Jorgensen of the State Serum Laboratory, Denmark, supplied samples of the F1 and He serotypes of VHS virus, and samples of IHN virus and herpesvirus salmonis were obtained from the ATCC. These samples were used as reference viruses.

The freeze dried virus samples which were supplied by various workers, or obtained from the ATCC were reconstituted with 1 ml MEM. A 1/10 dilution of the the reconstituted viruses were made. RTG2, BF2, FHM and BB cell lines were passaged and incubated at 22C for the RTG2 cells and 25C for the other cell lines until the cultures were 85-95 % confluent. The cultures were then inoculated according to the techniques described by Hill (1976a) and Wolf (1970). The culture medium was removed and 1 ml of the 1/10 dilution of the reconstituted viruses were added to the cultures. The cultures were incubated at 10C-15C for one hour to allow for the adsorption of the virus to the cells, after which the excess virus material was removed and maintenance medium consisting of MEM supplemented with 1% v/v NEAA and 2% v/v FCS, was added to the culture vessel. The cultures were then incubated at 10C-15C and observed daily for 14 days or until cytopathic effects (CPE) were seen. If CPE were seen, the culture fluids were stored as reference samples of the viruses. From then on, unless stated otherwise, all reference samples of the viruses were diluted 1/2000 before inoculation onto cells. The volume of the inoculum was dependent on the volume of the culture vessel. If 25 cm^2 flasks were used, 1 ml of the 1/2000 dilution of the reference virus was used. If 24 well plates were used, 100 µl of the 1/2000 dilution was used.

The different serotypes of IPN virus were stored at 4C after mixing equal volumes of the culture fluid with glycerol, as this improves the survival of IPN virus (Wolf, 1966; MacKelvie & Desautels, 1975). Samples of the other viruses were stored at -20C. Samples of all of the reference viruses were also stored in liquid nitrogen and by freeze drying with 50% buffered lactose phoshate (BLP). The freeze dried samples were stored at -20C. Samples of virus from each storage group were removed and inoculated onto cells by the methods described above. Freeze dried samples were also reconstituted by the addition of 1 ml Eagles MEM, and inoculated onto cells. This was done to check for survival of the viruses stored under different conditions.

3.3. Virus titrations.

The 50% tissue culture infective dose (TCID 50) of the stock viruses were calculated by the method of Luria, Darnell, Baltimore & Campbell (1978). Cells were grown in either the flat bottomed 96 well plates or 24 well plates. Ten-fold serial dilutions of the viruses were made in Eagles MEM and at least four wells were inoculated per dilution by the method described above. The cultures were incubated at 10C and the cells were observed daily for 7 days. The number of wells showing CPE per dilution was recorded. This information was then used to calculate the tissue culture infective dose (TCID 50) by a modification of the method described by Reed & Muench (1938).

If a particular dilution results in a total of 50% CPE, this dilution is used to calculate the TCID 50. If an exact 50% CPE is not achieved, the dilution which will give 50% CPE must be calculated. In this case, one dilution will result in more than 50% CPE, while the higher dilution will result in less that 50% CPE. These two dilutions must be identified and used in the calculation. The proportional distance between the two dilutions can be calculated according to the methods of Reed & Muench (1938).

Titres were calculated for a sample of each of the reference viruses according to the methods discussed above.

3.4. Plaque assay.

The procedures for plaquing fish viruses described by Wolf & Quimby (1973a; 1973b) were used. RTG2 cells were grown in 60 mm tissue culture quality petri dishes (Sterilab Services) and were inoculated with 200 µl of a 1/2000 dilution of the reference virus samples of the VR299, Ab and Sp serotypes of IPN virus once the cells were 85%-95% confluent. The cultures were incubated for 1 hour to allow for the adsorption of the virus to the cells, after which the inoculum was removed. Agar, at a 1% concentration was prepared in PBS, autoclaved and kept in a water bath at 40C until used. Equal volumes of double strength Eagles MEM with 20% FCS were added to the agar solutions in the water bath and incubated until used. The agar plus the 20 % FCS medium was

cooled to 39C, and 2 ml was poured onto the cells in the petri dishes. The agar was allowed to solidify and then overlayed with 1 ml of the Eagles MEM plus 2% v/v FCS to prevent it from drying out. The petri dishes were incubated at 15C in a CO_2 environment for seven days. After this incubation, the cells were fixed by adding 1 ml of a 40% formaldehyde solution to the cultures. The petri dishes were incubated for 10 mins. and the agar was removed by directing a stream of water at the junction between the agar and the petri dish. Once the agar had been removed the cell sheets were stained by flooding the petri dish with a 2% crystal violet solution.

A titre, expressed in plaque forming units (pfu) per ml could be calculated by counting the plaques per dilution.

3.5. Concentration of IPN virus

The methods used for the concentration of IPN virus were similar to those used by Kelly and Loh (1972). Samples of the VR299, Ab and Sp serotypes of IPN virus were inoculated onto RTG2 or BF2 cells and the cultures were incubated at 15C until good CPE were seen. The culture fluids were then centrifuged at 850 x g for 10 min. to remove all cell debris. The viruses were concentrated by ultra centrifugation at 78000 x g for 2 hours. The viruses were all resuspended in 0,5 ml PBS at a pH of 7.2.

3.6. Electron microscopy on IPN virus

Samples of the VR299 serotype of IPN virus were studied in the electron microscope by negative staining. Concentrated virus samples were used for the preparation of negatively stained grids for examination in the E.M. by the methods described by Kelly & Loh (1972) and Olberding & Frost (1975). One drop of the virus suspension was placed onto Fomvar coated grids and allowed to stand for 1 min. The excess fluid was removed with blotting paper and the grids were stained with 2% w/v phosphotungstic acid in water at a pH of 6.0 (Kelly & Loh , 1972) or 0.5% (w/v) phosphotungstic acid in water (Olberding & Frost, 1975). The excess fluid was removed, the grids were air-dried and then examined.

Attempts were also made to study IPN VR229 virus in infected cell cultures by scraping the cells from the culture vessel. The cells

were then centrifuged at 850 x g for 10 min. and the pellets were prepared for sectioning according to the methods described by Roberts (1980). Thin sections were cut and stained with a 20% w/v solution of uranyl acetate in absolute methanol and lead citrate (Roberts 1980).

3.7. Serological identification of IPN virus

3.7.1. Antisera

Samples of rabbit raised antisera against the Ab, Sp and VR 299 serotypes of IPN virus and antiserum against VHS virus were kindly supplied by Dr P. de Kinkelin. All samples of antiserum were freeze dried and stored at - 20C until used. Samples were reconstituted by adding 0,5 ml Eagles balanced salt solution (BSS) per vial. The antiserum was diluted 1/20 with Eagles BSS and stored in 1 ml quantities at -20C or in liquid nitrogen until use.

A group polyvalent antiserum against the VR299, Sp, and Ab serotypes of IPN virus was produced by mixing equal volumes of anti IPN VR299, anti IPN Ab and anti IPN Sp as most of the IPN viruses isolated from fish are serologically similar to one of these serotypes. The group polyvalent antiserum was stored in the same way as the other antiserum. In 1986, antiserum against the West Buxton serotype of IPN virus, which was supplied by Dr Hill, was added to the group polyvalent antiserum and this group polyvalent antiserum was used routinely there after.

Antibodies against the VR299 serotype of IPN virus, which was supplied by Dr Hill, were produced by firstly growing the virus on RTG2 cells. The titre was increased by passaging the virus twice in cell cultures and the culture fluid was purified by centrifugation at 850 x g for 10 min. to remove cellular debris. The titre, in TCID 50/ml was calculated according to the methods described above.

Antiserum was produced in female New Zealand white rabbits by a method similar to the one described by Hill *et al* (1981). The technique used to produce antisera differed from those used by Hill, *et al* (1981) in that the virus suspension was not purified

or concentrated by ultracentifugation. The rabbits were inoculated into the ear vein which corresponds to one of the techniques used by Hill et al (1981). The presence of the viral specific antibodies in the rabbit serum was demonstrated by the virus neutralization test (Hill, 1976a).

3.7.2. Serological tests

The different serotypes of IPN virus were identified by the indirect fluorescent antibody test (Hill, 1976a; Piper et al, 1973), indirect immunoperoxidase test (Nicholson & Henchal, 1978), serum neutralization test (Hill, 1976a; Lientz & Springer, 1973), viral induced bacterial co-agglutination test (Kimura et al, 1984) and the direct and indirect immunostaphylococcus-protein-A (ISPA) test (Bragg & Combrink, 1987b).

3.7.2.1. Indirect immunofluorescent antibody test

The antisera used for the indirect immunofluorescent antibody test were anti IPN VR299, anti IPN Ab, anti IPN Sp, and a group polyvalent antiserum against the VR299, Ab and Sp serotypes of IPN virus (all supplied by Dr de Kinkelin). All of these antibodies were rabbit raised and commercially available FITC labelled anti-rabbit was used.

The procedures for the indirect immunofluorescent antibody technique were similar to those used by Hill (1976a). Cultures of RTG2 cells were established in tissue culture quality petri dishes with a diameter of 60mm containing glass cover slips as discussed by Hill (1976a). Reference samples of the different serotypes of IPN virus and VHS virus were diluted 1/2000 with MEM and 200 µl of these dilutions were inoculated onto the cell cultures once the cells were 85% - 95% confluent and incubated at 15C until CPE developed. The cells were then fixed with acetone for ten minutes and washed twice with PBS.

Antisera were diluted 1/20 and 0.2 ml was added to the cells and incubated for five to seven minutes in a moist environment. The cells were then washed three times by flooding with PBS. Samples of a 1/100 dilution of the FITC labelled goat anti-rabbit antiserum was added to the cells and incubated for five to seven minutes in a moist chamber. The cells were then washed three times by flooding with PBS, air-dried and studied under a microscope with a mercury light source.

Uninfected cells and cells infected with VHS virus and processed according to the methods discussed above, were used as negative controls.

3.7.2.2. Indirect immunoperoxidase test

The methods for the indirect immunoperoxidase test were similar to those used by Nicholson & Henchal (1978). Cultures of RTG2 or BF2 cells were passaged and used to seed 60 mm petri dishes containing glass cover slips which were incubated until 85% confluent. The cells were then inoculated with samples of the different serotypes of IPN virus and incubated until CPE developed. The cells were then fixed with acetone for ten minutes and washed twice with PBS. A 1/20 dilution of the virus specific antiserum was prepared and 0.2 ml was added to the cells and incubated for five to seven minutes in a moist environment. The cells were then washed three times by flooding with PBS. Approximately 0,2 ml of a 1/100 dilution of the peroxidase labelled goat anti-rabbit antiserum was added to the cells and incubated for five to seven minutes in a moist chamber. The cells were then washed three times by flooding with PBS. A saturated solution of 3,3 diaminobenzidine tetrahydrochloride (in 0.05 M tris-HCl buffer, pH 7.6, which contained 0.01% hydrogen peroxide) was added to the cell sheet and allowed to react for 10 mins. The cultures were washed and studied under the microscope. A brown precipitate on the cell sheet was an indication of the presence of viral antigens in the cells.

As with the indirect immunofluorescent antibody test, uninfected cells and cells infected with VHS virus and processed as above were used as negative controls.

3.7.2.3. Serum neutralization tests

The serum neutralization test was carried out according to the procedures described by Hill (1976a) and Lientz & Springer (1973). This test was performed by first passaging cultures of RTG2, FHM or BF2 cells by the above methods and using this to seed 24 well plates. These plates were incubated until 85% confluent and then used for the serum neutralization test. Samples of VHS virus and the serotypes of IPN virus were then diluted 10-fold from a 10^{-1} dilution to a 10^{-6} dilution. Samples of anti IPN VR299, anti IPN Ab, anti IPN Sp and a group polyvalent antiserum against all three serotypes of IPN virus were diluted to 1/10 or 1/100 and equal volumes of the diluted antiserum were mixed with each sample of the diluted virus and the mixtures were incubated at 20C for 60 min. Virus controls were set up by mixing equal volumes of a 1/1000 dilution of FCS with each sample of the diluted virus and incubating them at 20C for 60 min. Duplicate wells of 24 well plates were inoculated with 0.2 ml of the virus/antiserum or virus/FCS complexes according to the above methods. The plates were then incubated at 15C in a CO_2 environment and examined daily for 7 days for the development of CPE. Neutralization of the viruses, as seen by the absence of CPE in the wells inoculated with the virus/antiserum mixture, could be observed. CPE had to be observed in the wells inoculated with the virus/FCS combination to confirm the test.

The neutralization index was calculated as the log of the difference between the titre of the virus with FCS and the virus with the test serum. If there was complete neutralization, the neutralization index was regarded as being greater than six. A neutralization index of one was regarded as negative and a neutralization index of 1,7 or more was positive (Casals, 1967).

Samples of VHS virus and antisera against the different serotypes of IPN virus were mixed and inoculated onto cells as negative controls.

3.7.2.4. Direct immunostaphylococcus-protein-A (ISPA) test

Samples of Staphylococcus aureus Cowan strain A were sensitized according to the methods described by Kimura et al (1984). Freeze dried cultures of S. aureus Cowan strain A were obtained from the Section of Bacteriology of the Veterinary Research Institute, Onderstepoort. These samples were reconstituted and plated onto blood tryptose agar (BTA) plates and incubated for 24 hours at 37C. A Gram stain was done on the cultures to check for purity.

Trypticase soy broth was inoculated with samples of the bacterium and incubated at 37C for 24 hours. The cultures were centrifuged at 1000 x g for 10 min., resuspended in PBS and washed three times. The pellet was resuspended in a 10% (v/v) solution of formaldehyde in water and incubated for 1 hour at room temperature to inactivate the bacteria. The bacterial suspension was then washed three times and resuspended in PBS to a final volume of approximately 10 times the pact cell volume (PCV) of the bacteria. The inactivation of the bacterial suspension was then confirmed by plating onto BTA plates and incubating the plates at 37C for 24 hours.

Samples of the antisera used for binding to the Protein A produced in the cell membrane of S. aureus Cowan strain A, were supplied by Dr de Winkelin. The virus specific antibodies were bound to the bacteria by the technique described by Hodnichak, Turley-Shoger, Mohanty & Rosenthal (1984). Approximately 0,5.ml samples were mixed with two ml of the inactivated bacteria. The suspension was incubated at room temperature for one hour and centrifuged at 1000 x g for 10 min.

Before the direct ISPA test could be done, the binding of the antibodies to the Protein A of S. aureus Cowan strain A was checked by doing an immunofluorescent or immunoperoxidase test. Samples of inactivated S. aureus Cowan strain A which had been sensitized with rabbit raised antibodies were placed onto glass slides and heat fixed. Samples of inactivated S. aureus Cowan strain A which were not sensitized were also placed onto clean glass slides and were heat fixed as negative controls. The slides containing both the sensitized and unsensitized bacterial smears were overlayed with 0.02 ml of a 1/1000 dilution of commercially available FITC labelled goat anti-rabbit antiserum. The slides were incubated in a moist chamber for five to seven min. and were washed three times with PBS. The slides were examined under a microscope with a mercury light source. In the immunoperoxidase test, both sensitized and unsensitized bacteria were placed onto glass slides. The bacteria were heat fixed and 0.02 ml of a 1/1000 dilution of commercially available peroxidase labelled goat anti-rabbit antiserum was added to both the sensitized and unsensitized bacteria. The slides were incubated in a moist chamber for five to seven min. and were then washed three times

with PBS. The substrate, 3,3 diaminobenzidine tetrahydrochloride with 0.01 % hydrogen peroxide was added to the glass slides and incubated for 10 min. The slides were washed well with PBS and examined under a microscope. These experiments were repeated using FITC labelled goat anti-mouse and peroxidase labelled goat anti-mouse.

Once the antibodies had been bound to the Protein A in the membrane of S. aureus Cowan strain A, and this binding had been confirmed by either the immunoperoxidase or immunofluorescent assay, the direct IPSA test could be carried out. The method for the direct immunostaphylococcus-protein-A test was the same as described by Bragg & Combrink (1987b). Cultures of RTG2, FHM or BF2 cells were passaged and used to seed petri dishes which were incubated until 85% confluent. The cells were then inoculated with samples of the reference viruses, and incubated until CPE developed. The cells were fixed with cold acetone (-20C) for ten minutes and washed twice with PBS. The direct ISPA test was done by adding 0,2 ml of the viral specific antibody sensitized S. aureus Cowan strain A to the petri dishes and incubating for five to seven mins in a moist environment. The petri dishes were washed three times by flooding with PBS. The cells were stained by adding 1 ml May-Grunwald stain and incubated for 10 mins. After 10 mins this stain was removed and 1 ml of a 1/10 dilution of Giemsa stain in buffered water, was added to the cells and incubated for 15 min. the stain was washed off and the cell sheets were allowed to air-dry. The cell sheets were examined under the 100x objective of a microscope and the number of attached bacteria per cell were counted. A random selection of 10 fields of view were used to calculate the average number of attached bacteria per cell.

Cells which were not infected with any virus were processed in the same way as described above. These cell cultures served as the negative controls.

3.7.2.5. Indirect immunostaphylococcus-protein-A (ISPA) test

Samples of S. aureus Cowan strain A were processed in the same way as described above. The bacteria for this test were sensitized with goat anti-rabbit serum by the above method. The method for the indirect ISPA test were the same as described by Bragg & Combrink (1987b). The cells were inoculated, incubated and fixed in the same way as for the direct method. Once the cells were fixed, 0,2 ml of a 1/200 dilution of the rabbit raised viral specific antiserum was added to the petri dishes and incubated for five to seven mins in a moist environment. The petri dishes were then washed three times by flooding with PBS

and 0,2 ml of the goat anti-rabbit sensitized S. aureus Cowan strain A was added to each petri dish and incubated for five to seven min. in a moist environment. The petri dishes were washed three times by flooding with PBS, and stained by the method used for the direct ISPA test. The stain was washed off and the cell sheets were allowed to air-dry and the average number of attached bacteria per cell were counted according to the method described above. Negative control cells were not infected with any virus but were processed in the same way as described above.

3.7.2.6. Viral induced bacterial co-agglutination (VIBCA).

The viral induced bacterial co-agglutination test was done according to the methods described by Kimura et al (1984). Samples of the inactivated S. aureus Cowan strain A were sensitized with anti IPN VR299, anti IPN Sp, anti IPN Ab, or anti VHS by the methods described above. A 10 µl sample of the anti IPN VR299 sensitized S. aureus Cowan strain A was placed on a clean glass slide and mixed with 10µl IPN VR299 virus (titre of 4.065×10^6 TCID 50/ml). The slide was rotated for one min. to facilitate the mixing of the virus dilution and the bacteria/antibody complex. The slide was examined for the formation of clumps of bacteria. The VIBCA test was also carried out by mixing either IPN Sp virus, IPN Ab virus or VHS virus with S. aureus Cowan strain A which had been sensitized with antiserum homologous to the virus.

The VIBCA test was also carried out by mixing 10 µl of anti VHS sensitized S. aureus Cowan strain A with 10 µl of culture fluid from cultures infected with each of the serotypes of the IPN viruses which showed good CPE. Negative controls consisted of mixing 10µl of sensitized S. aureus Cowan strain A with 10 µl of PBS in the same way as discussed above, or by mixing 10 µl of the different viruses with 10 µl of unsensitized S. aureus Cowan strain A.

3.8. Comparison of the different serological techniques for the identification of IPN virus.

A culture of RTG2 cells was inoculated with a 1 ml sample of IPN VR299 virus by the method described above and incubated at 15C until CPE were seen. The culture fluid was removed and the titre was calculated in TCID 50/ml, by the method described above. Ten fold serial dilutions, from 10^{-1} to 10^{-10}, of the culture fluid were made in Eagles MEM. Samples of each dilution were used to do the VIBCA test, the serum neutralization test and to inoculate

three 95% confluent cultures of RTG2 cells in 60 mm diameter petri dishes. Another three petri dishes were left as negative controls. The petri dishes and the serum neutralization test were incubated at 15C and observed daily. As soon as CPE were detected in the petri dishes, the direct ISPA, indirect ISPA and indirect immunofluorescent antibody test were performed in the dishes by the methods discussed above. Controls on these tests were done on the uninfected cells. The highest dilution of the culture fluid which could be used to obtain positive results by the different tests was recorded.

3.9. Isolation and identification of virus from diseased fish.

Samples of diseased fish for the isolation of viruses were submitted to the Fish Disease Unit of the Veterinary Research Institute, Onderstepoort. These fish came from populations which were showing abnormalities such as exopthalmus, darkening of the skin, erratic swimming, accumulation at the water outflows and high mortality rates.

The techniques for the isolation of viruses from fish have been discussed by Wolf (1970) and reviewed by Hill (1976a) and Amos (1985) and these techniques were followed.

Fish were placed on tissue paper and the skin surface sterilized by washing with methyolate. The skin and muscle layers were cut from the vent to the mouth and the body wall on one side was cut off. Samples of the liver and spleen were removed and placed into 9 ml sterile Eagles MEM. The gut was removed to reveal the kidney, which was also removed and added to the other organs in the medium. If a number of fish were submitted, they were pooled into groups of five (Hill, 1976a).

The organ samples were homogenized in a laboratory homogenizer and were diluted 1/10 with Eagles MEM. The samples were filtered through a 0,45 µm membrane filter to remove bacterial contamination (Hill 1976a) and strict sterile procedures were thus not essential during the collection of samples.

Samples were inoculated onto duplicate wells of RTG2, BF2 or FHM cells by the methods discussed above and samples were incubated at 15C for 14 days (Amos, 1985) and examined daily for the development of CPE. If no CPE were seen to develop after 14 days, all the culture fluids were passaged onto fresh cell cultures and incubated for a further 14 days as it has been found that in some cases CPE only develop after passaging (Hill, 1976a). In 1986, the procedure was changed to an incubation period of 7

days as recommened in Hill (1976a), followed by passage and incubation for a further 7 days was used routinely in this laboratory. If CPE developed, the culture fluid from those cultures showing CPE were removed, diluted 1/10 and inoculated onto fresh cell cultures. This was done to ensure that the CPE were due to the presence of a virus and not due to toxicity of the sample (Amend & Wedemeyer, 1970). If CPE developed again, the culture fluid was harvested and the serum neutralization test, immunofluorescence test or the direct ISPA test was done by the methods described above.

3.10. Isolation and identification of viruses from asymptomatic fish.

Asymptomatic fish for the isolation of viruses were sampled during the normal slaughtering operations on the farms, thus making the collection of large numbers of viscera samples possible. It is never possible to test 100% of the fish in a population, thus statistically representative samples must be collected. Simon & Schill (1984) presented a number of tables of sample sizes to detect various incidences of carriers in a population with varying degrees of confidence. It was decided to try and detect a 2% incidence of a disease with 95% confidence. The number of samples to be collected to achieve this was taken from the tables presented by Simon & Schill (1984). Methods for the collection and processing of viscera samples were the same as used for the processing of samples from diseased fish as discussed above. The cells were inoculated and incubated at 15C as discussed above and the culture fluid from any culture showing CPE after passage was used for the serum neutralization, indirect fluorescent antibody test, and the direct ISPA test which were done by the methods described above.

3.11. Isolation and identification of virus from ovarian fluid of asymptomatic fish.

As in the case of isolation of viruses from viscera of asymptomatic fish, it was decided to try and detect a 2% incidence of carriers with 95% confidence and to this end, statistically representative samples of ovarian fluid were collected according to the tables presented by Simon & Schill (1984). Samples of ovarian fluid were collected during the stripping operations on the farms. Five female rainbow trout were stripped into a sieve over a bowl. The ova are retained by the sieve and the ovarian fluid collected. The samples were filtered

through a 0,45 µm membrane filter and inoculated onto duplicate wells of RTG2, BF2 or FHM cells by the methods discussed above. Samples of ovarian fluid were not diluted before addition to the cell cultures as undiluted ovarian fluid is not toxic to the cells (Amend & Wedemeyer, 1970). The 24 well plates were then processed in the same way as described for the isolation of viruses from diseased fish. If any viruses were isolated, they were identified by the serum neutralization test, immunofluorescent antibody test and the direct ISPA test according to the methods set out above.

3.12. Isolation and identification of viruses from eyed ova.

Wolf, et al (1963) discussed the techniques for the isolation of viruses from trout ova and these methods were followed. Ten ova were placed into 9 ml Eagles MEM and the ova were disrupted with sterile tweezers. The medium plus the disrupted ova were diluted 1/10 in Eagles MEM and filtered through a 0,45 µm membrane filter. The filtrate was used to infect susceptible cell cultures and the plates were handled in the same way as described above. Samples of virus isolated from eyed ova were identified by the serum neutralization test, indirect immunofluorescent antibody test and the direct ISPA test according to the methods set out above.

3.13. Detection of antibodies in the serum of fish.

Blood samples were collected from diseased fish submitted to the Fish Disease Unit, while blood samples from asymptomatic fish were collected on the farms.

Blood was collected from rainbow trout by cardiac puncture with a 25 gauge needle. The blood was placed in 1 ml tubes and incubated at room temperature for about 15 mins. The clot was loosened and the serum removed.

The collected serum was used in a virus neutralization test according to the procedures described by Wolf & Quimby (1969). The serum samples were serially diluted from 10^{-1} to a 10^{-6}. The virus neutralization test was performed by passaging cultures of RTG2 or BF2 cells by the above methods and using them to seed 24 well plates. These plates were incubated until 85% confluent and then used for the virus neutralization test. Samples of IPN VR299 virus and VHS virus were diluted to a dilution of 1/100 or 1/1000 depending on the titre. Equal volumes of the diluted serum and

the diluted trout viruses were mixed and were incubated at 20C for 60 min, with periodic shaking. Positive controls were set up by mixing equal volumes of the diluted viruses with dilutions of virus specific antibodies. Negative controls were set up by mixing samples of the reference viruses with diluted FCS. Duplicate wells of 24 well plates were inoculated with 0.2 ml of the fish serum/ virus mixture, positive controls and negative controls by the above methods. The plates were then incubated at 15C in a CO_2 environment and examined daily for the development of CPE.

the diluted trout viruses were mixed and were incubated at 20C for 60 min, with periodic shaking. Positive controls were set up by mixing equal volumes of the diluted viruses with dilutions of virus specific antibodies. Negative controls were set up by mixing samples of the reference viruses with diluted FCS. Duplicate wells of 24 well plates were inoculated with 0.2 ml of the fish serum/ virus mixture, positive controls and negative controls by the above methods. The plates were then incubated at 15C in a CO_2 environment and examined daily for the development of CPE.

4. RESULTS

4.1. Tissue culture

Samples of the RTG2 cells (Wolf & Quimby, 1962), BF2 cells (Wolf et al, 1964), FHM cells (Gravell & Malsberger, 1965) and BB cells (Cerini & Malsberger, 1962) were obtained from the ATCC. Some cultures were supplied as frozen cultures and these were successfully reconstituted. Other samples were reconstituted by the supply company and were supplied as confluent cultures. All the cultures were passaged and propagated by the methods described above and the results recorded (Table 1).

Table 1. Results of the passage and propagation of four fish cell lines.

Cell line	No. pass[1]	New[2]	Succ.[3]	Unsucc.[4]	Highest[5] Pas. No.	Success Rate (%)
RTG2	292	1050	792	258	89	75,42
FHM	402	1082	744	338	99	68,76
BB	147	369	240	129	139	65,04
BF2	272	718	640	78	52	89,14

1 = Number of cultures passaged
2 = Number of new cultures established
3 = Number of new cultures which successfully reached 100% confluency
4 = Number of cultures discarded.
5 = Highest Passage Number

All cell cultures were examined daily and the area covered by the cells was recorded as percentage growth. From these results the time taken by each culture to completely cover the surface of the flask can be obtained. These results are represented in Table 2 and Fig 5 - 8.

Samples of all four cell lines were stored by freezing in liquid nitrogen by the methods described above. Samples of these cells were also reconstituted as described and the results recorded (Table 3).

Table 2. Incubation periods for the four fish cell lines to reach 100% confluency.

Days to 100% confluency	Number of cultures			
	RTG2	BF2	FHM	BB
1	-	28	44	6
2	7	142	207	37
3	39	221	141	37
4	92	105	131	48
5	141	89	59	47
6	202	44	71	24
7	147	2	15	11
8	46	4	31	10
9	13	4	8	9
10	26	-	20	1
11	23	-	2	6
12	16	-	13	6
13	9	-	-	7
14	18	-	-	-
15	1	-	-	1
16	6	-	2	-
17	-	-	-	-
18	6	-	-	-
Average[1]	6,58	3,42	3,98	5,23

1: Average IP in days for a particular cell line is calculated by multiplying the number of cultures by the days to confluency, then adding all these figures and dividing by the total number of successful cultures.

Table 3. Results of the freezing and reconstitution of the four fish cell lines.

Cell line	Number frozen	Number recon-stituted	Success-ful	Unsuccess-ful	Success rate (%)
RTG2	52	24	11	13	45,8
FHM	30	19	19	0	100,0
[illegible][2]	27	10	7	3	70,0
BF2	75	44	29	15	65,9

FIG. 5 GRAPH FOR RTG2 CELLS
DAYS TO CONFLUENCY VS NO. OF CULTURES

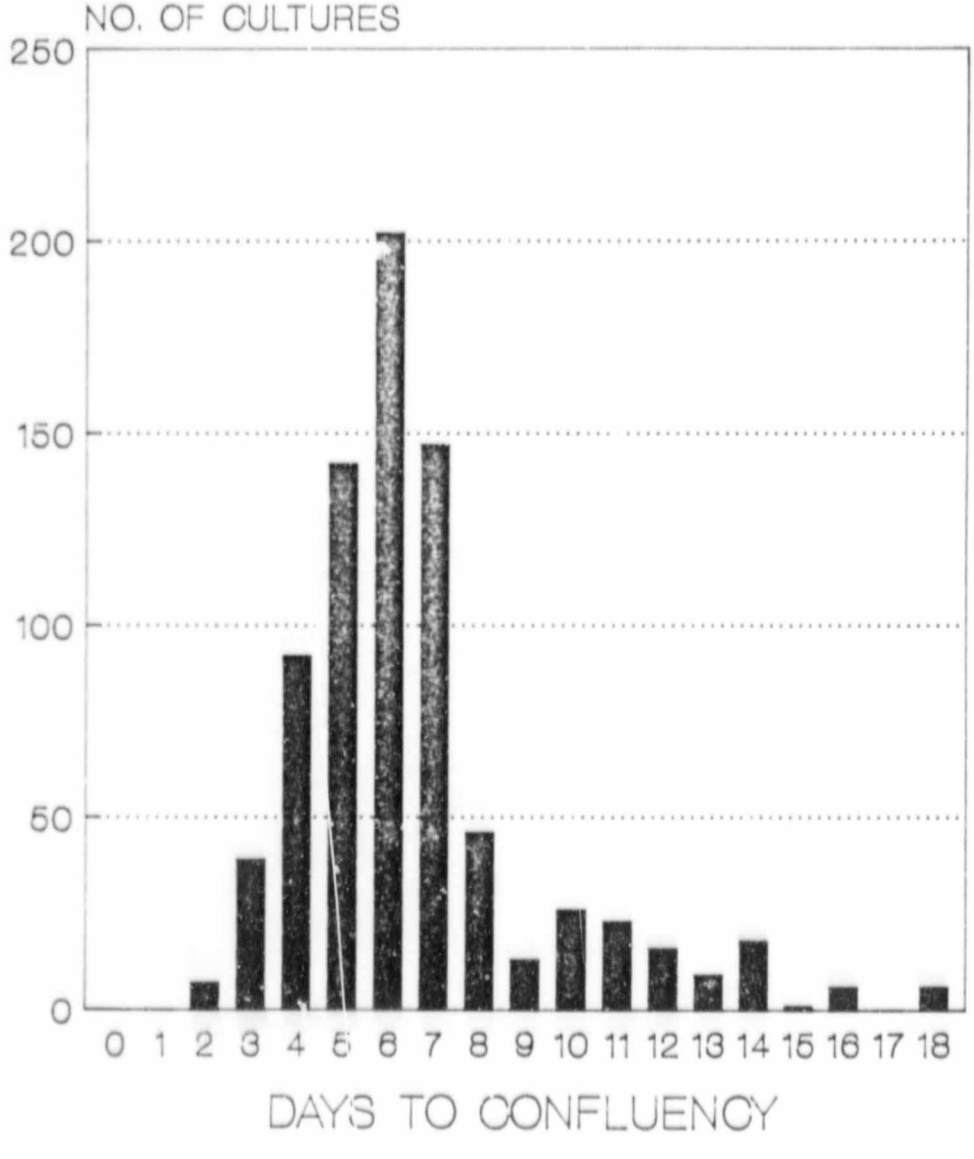


FIG. 6 GRAPH FOR FHM CELLS
DAYS TO CONCLUENCY VS NO. OF CULTURES

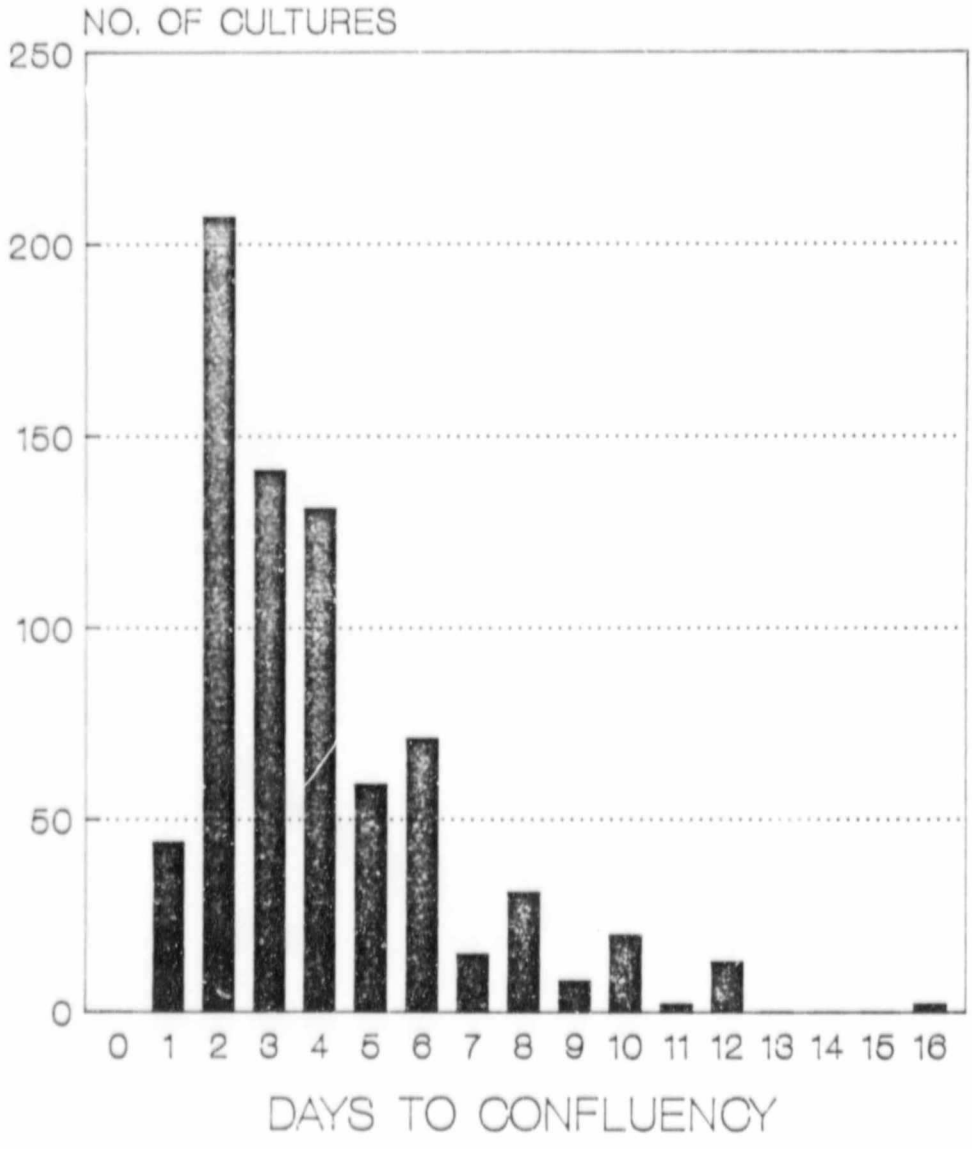


FIG. 7 GRAPH FOR BB CELLS
DAYS TO CONFLUENCY VS NO. OF CULTURES

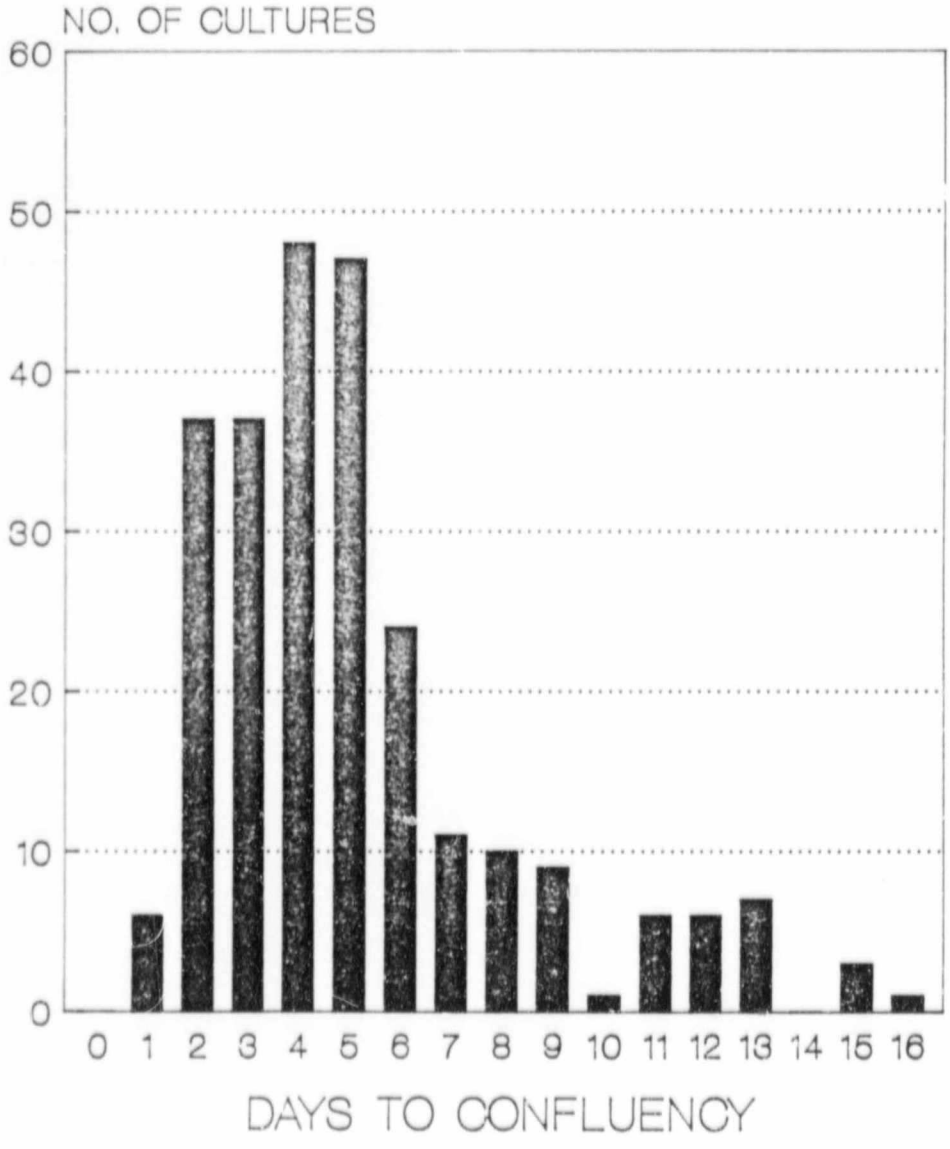


FIG. 8 GRAPH FOR BF2 CELLS
DAYS TO CONFLUENCY VS NO. OF CULTURES

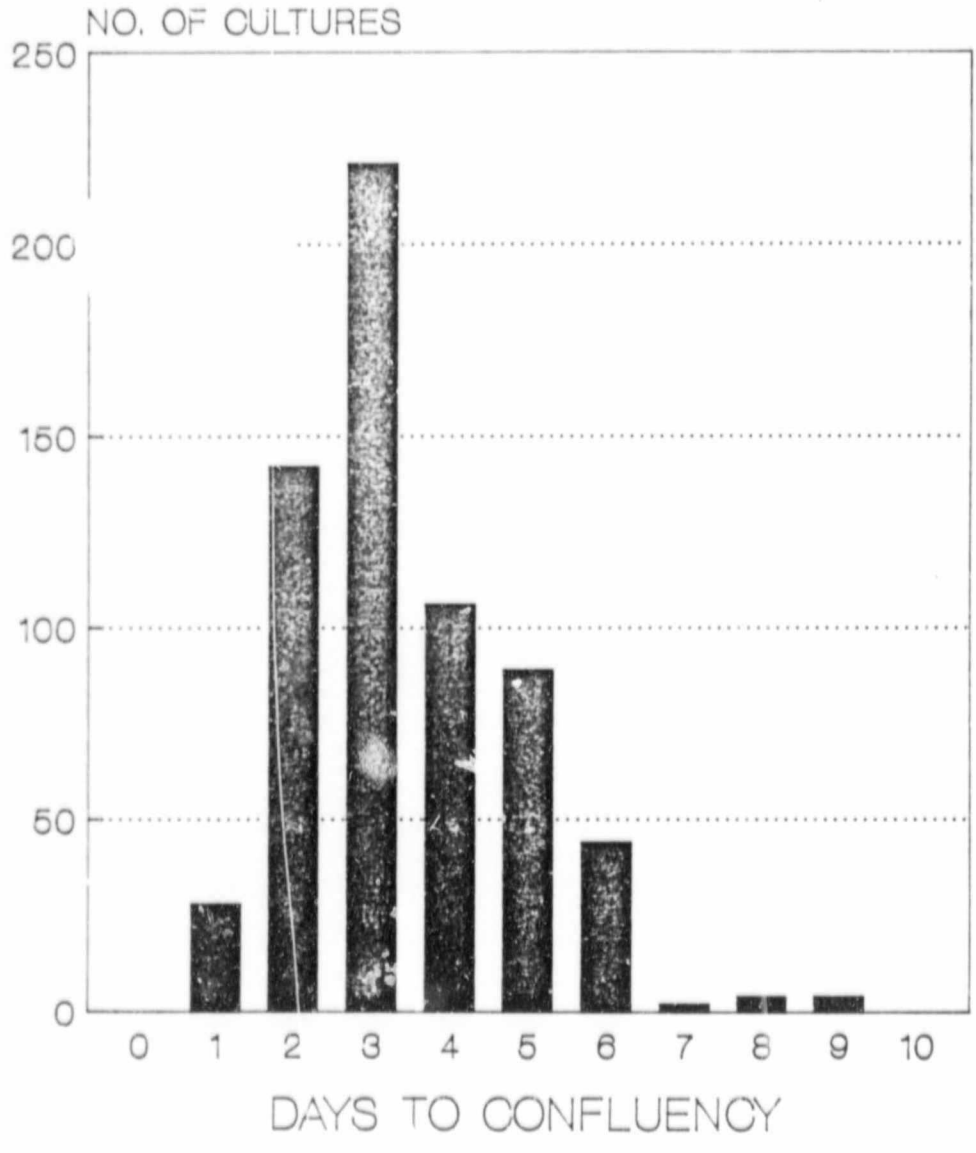
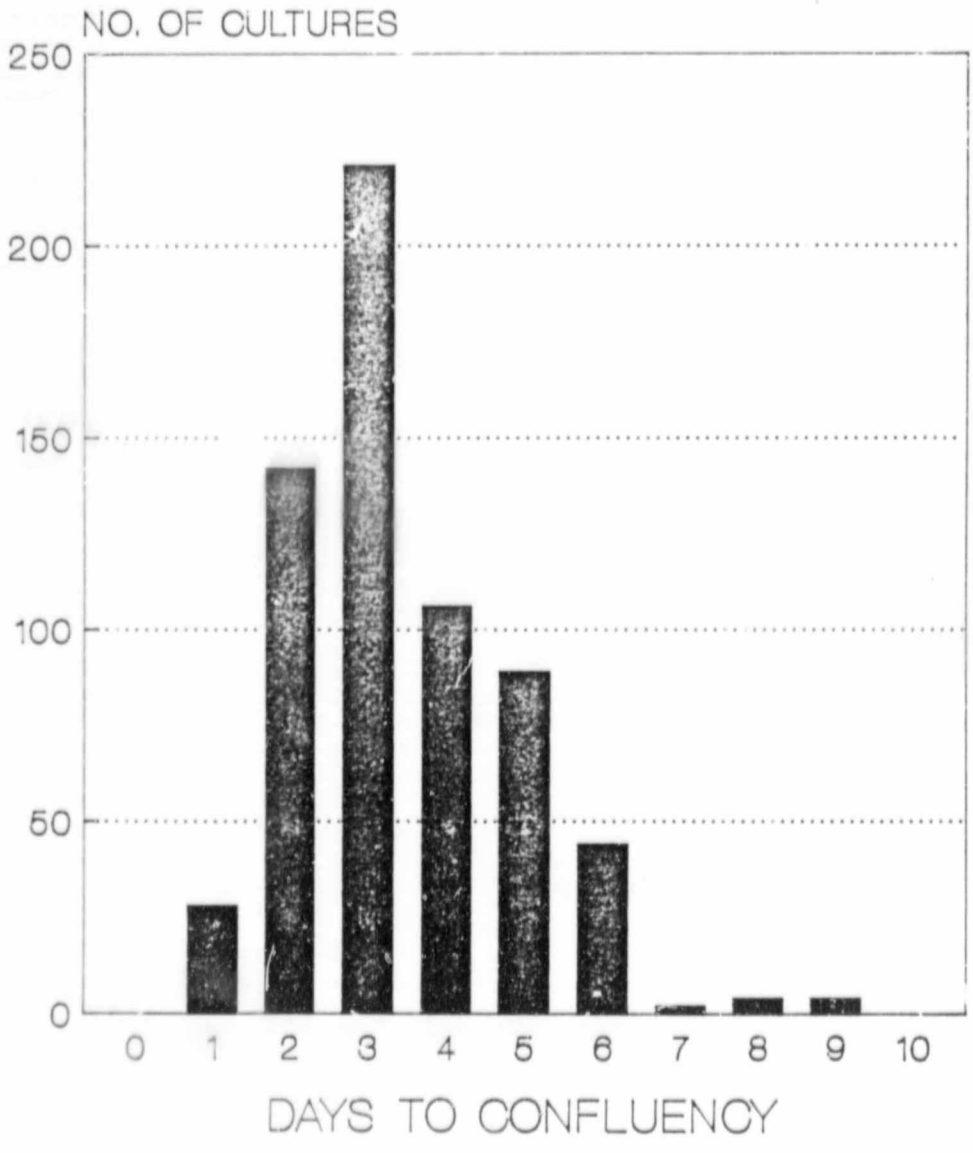


FIG. 8 GRAPH FOR BF2 CELLS
DAYS TO CONFLUENCY VS NO. OF CULTURES



4.2. Virus inoculation

The cytopathic effects (CPE) and incubation periods (IP) resulting from the inoculation of the RTG2, BF2, FHM and BB cell lines with samples of the VR299, Ab and Sp serotypes of IPN virus, IHN virus, VHS virus and herpesvirus salmonis were recorded (Table 4). It was found that cells with CPE were quite distinct from normal uninfected cells which form a confluent monolayer (Fig 9). The VR299 and Sp serotypes of IPN virus produced CPE on all four of the cell lines in use. IPN Ab virus did not produce CPE on FHM cells but did so on the other cell lines. It was found that the different serotypes of IPN virus produced similar CPE on a particular cell line. The CPE produced by IPN virus were however, different on the different cell lines (Fig 10). IHN virus and VHS virus were found to produce CPE on the RTG2, BF2 and FHM cell lines (Fig 11 & Fig 12), while herpesvirus salmonis was found to grow only on the RTG2 cell line with typical CPE (Fig 12D).

The cell lines of choice for the isolation of the different trout viruses were determined from the average IP and the success rates obtained when the cell lines were inoculated with the different viruses (Table 4). In all cases, the success rates when using FHM cells were very low, with the highest being 50% obtained when this cell line was inoculated with IHN virus. These low success rates exclude the use of this cell line when attempting to isolate viruses from trout in South Africa. The BB cell line had good success rates when the different serotypes of IPN virus were inoculated onto the cells. The BB cells were, however, refractory to VHS virus, IHN virus and herpesvirus salmonis. The average IP for the development of CPE on BB cells were very long, thus making this line unsuitable for the isolation of viruses from trout. The two remaining cell lines (RTG2 and BF2) both have acceptable success rates and low IP, thus these two are the lines of choice when attempting to isolate viruses from trout.

Table 4. Results obtained from the inoculation of the fish cell lines with samples of the reference viruses.

Cells	Virus	Total	Positive	Negative	% success	Average IP[1] (Hours)
RTG2	IPN VR299	24	15	9	62,5	46
BF2	IPN VR299	17	14	3	82,4	62
FHM	IPN VR299	8	2	6	25,0	72
BB	IPN VR299	5	4	1	80,0	120
RTG2	IPN Ab	13	9	4	69,2	57
BF2	IPN Ab	7	5	2	71,4	58
FHM	IPN Ab	4	0	4	-	-
BB	IPN Ab	3	2	1	66,5	72
RTG2	IPN Sp	11	8	3	72,7	48
BF2	IPN Sp	12	7	5	58,3	58
FHM	IPN Sp	11	3	8	27,3	72
BB	IPN Sp	4	4	0	100,0	96
RTG2	IHN	19	19	0	100,0	49
BF2	IHN	15	15	0	100,0	53
FHM	IHN	10	5	5	50,0	77
BB	IHN	3	0	3	-	-
RTG2	VHS	13	13	0	100,0	39
BF2	VHS	13	13	0	100,0	72
FHM	VHS	11	5	6	45,5	43
BB	VHS	3	0	3	-	-
RTG2	Herpesvirus salmonis	8	7	1	87,5	58
BF2	Herpesvirus salmonis	4	0	4	-	-
FHM	Herpesvirus salmonis	2	0	2	-	-
BB	Herpesvirus salmonis	3	0	3	-	-

1 = Average incubation period for the development of CPE.

Fig 9. Normal, uninfected cells. A) RTG2 cells B) BF2 cells C) FHM cells and D) BB cells.

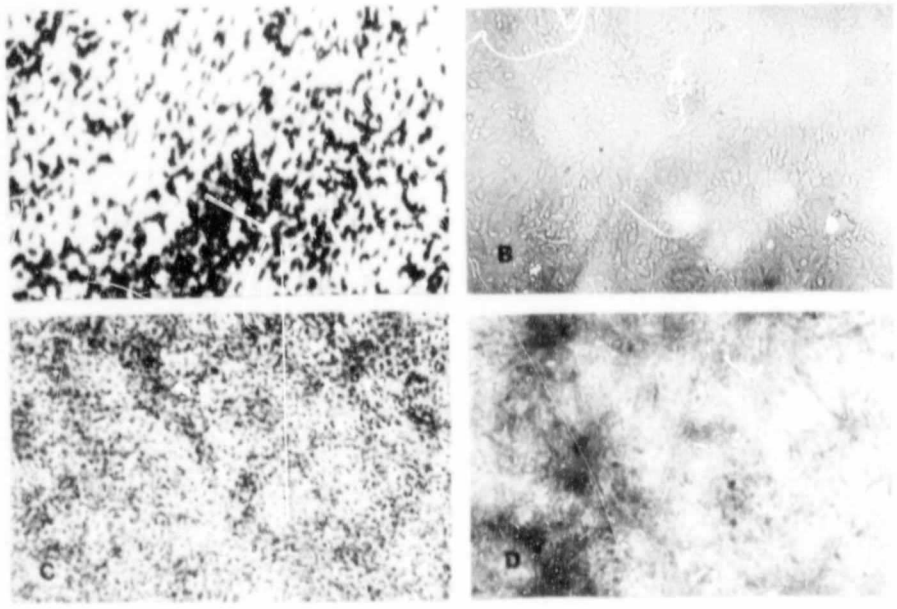


Fig 10. CPE caused by IPN virus on A) RTG2 cells, B) BF2 cells, C) FHM cells and D) BB cells