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ISOLATION AND IDENTIFICATION OF TROUT VIRUSES IN SOUTH AFRICA

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ABSTRACT

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A total of 3 257 samples of diseased rainbow trout were examined for the presence of viruses from January 1983 to December 1987. A virus closely related to the VR 299 serotype of infectious pancreatic necrosis virus was isolated from 13 cases. An additional 7 228 viscera samples from asymptomatic fish were collected during the same period and a similar virus was isolated from 2 sites. During the same period 2 892 ovarian fluid samples were collected and a similar virus was isolated from 1 site. A similar virus was also isolated from one consignment of imported trout ova. A total of 5 550 ova was examined during this period. The viruses were identified by various tests as being closely related to the VR299 serotype of infectious pancreatic necrosis virus. All these samples tested negative for infectious haematopoietic necrosis virus, viral haemorrhagic septicaemia virus and herpesvirus salmonis.

INTRODUCTION

The first successful introduction of rainbow trout (*Salmo gairdneri* Richardson 1938) into South Africa was in 1897 (McC. Pott, 1987). Since that time trout have become well established in all of the provinces and a total of about 520 tons of trout was produced in 1985 (McC. Pott, 1987).

The first isolation of a virus from fish was in 1957 (Wolf, Snieszko, Dunbar & Pyle, 1960) when infectious pancreatic necrosis virus (IPN) was isolated from rainbow trout. Since this isolation, viruses have been isolated from a wide range of fish and a viral aetiology has been established for 17 diseases (Wolf & Mann, 1980; Wolf, 1982). A further 15 diseases are suspected of having a viral aetiology (Wolf, 1982).

The most important viral diseases of rainbow trout are IPN, infectious haematopoietic necrosis virus (IHN) and viral haemorrhagic septicaemia virus (VHS). Herpesvirus salmonis was isolated from trout but this virus appears to be limited to some hatcheries in the USA and Japan (Wolf, 1976; Sano, 1976).

IPN has an almost worldwide distribution and has been isolated in North America (Wolf *et al.*, 1960), Europe (Besse & De Kinkelin, 1965), Japan (Sano, 1971), South America (McAllister & Reyers, 1984), Korea (Hedrick, Eaton, Fryer, Hah, Park & Hong, 1985), Taiwan (Hedrick, Fryer, Chen & Kou, 1983) and South Africa (Bragg & Combrink, 1987b). IHN, on the other hand, has been isolated in the USA (Amend, 1974) Japan (Sano, Okamoto, Nishimura, Yamazaki, Hanada & Ularanaba, 1977) and Europe (Bovo, Giorgetti, Jorgensen & Olesen, 1987). VHS has been isolated only in Europe (Wolf, 1972). There is a report of VHS in South Africa (Lombard, 1968) but no virus could be isolated and the diagnosis was based on symptoms alone. That infected fish responded to treatement with antibiotics and vitamins (Lombard, 1968) indicates that this disease was of bacterial rather than viral aetiology.

The characteristics of all the viruses isolated from fish, including IPN, IHN, VHS and herpesvirus salmonis have been comprehensively reviewed by Pilcher & Fryer (1980).

The demand for the virological examination of rainbow trout in South Africa stems from the demand for certified disease-free trout ova in the Northern Hemisphere. The reason for the demand for ova from the Southern Hemisphere is due to the fact that trout breed only during the winter and if a constant supply of market size fish is to be maintained, ova must be obtained during the summer. The most practical source of "summer ova" is the Southern Hemisphere. The fact that the viral diseases can be egg-transmitted (Bullock, Rucker, Amend, Wolf & Stuckey, 1976) makes the virological examination of the brood stock essential.

The present report represents the results of a diagnostic survey carried out from January 1983 to December 1987 on samples of diseased fish, trout ova and statistically representative viscera and ovarian fluid samples from asymptomatic fish, and is aimed at the certification of ova for export.

MATERIALS AND METHODS

Cell culture

Samples of the RTG2 (Wolf & Quimby, 1962), BF2 (Wolf & Quimby, 1966) and FHM (Gravell & Malsberger, 1965) cell lines were obtained from the American Type Culture Collection (ATCC). The cells were passaged and propagated according to the methods described by Wolf & Quimby (1978) in sterile 24-well tissue culture plates[1].

Sample collection

Samples of diseased trout were submitted to the Fish Disease Unit, where samples of the liver, spleen and kidney were removed and placed in sterile phosphate buffered saline (PBS), containing 240 mg of penicillin/ℓ and 400 mg of streptomycin/ℓ (Hill, 1976; Amos, 1985). Samples of eyed ova were collected from local brood stocks or from imported ova in PBS plus antibiotics.

Statistically representative samples of liver, spleen and kidney from asymptomatic fish were collected according to the tables presented by Simon & Schill (1984). An attempt was made to detect a 2 % incidence of a disease with 95 % confidence and, to this end, 150 samples were collected from all sites with a population greater than 100 000 fish. These samples were collected on the farms in sterile PBS plus antibiotics. The samples were pooled into groups of 5 fish per pool. Ovarian fluid was also collected from asymptomatic brood fish. Groups of 5 females were stripped into a sieve and the fluid was collected in a bowl. A 10 mℓ sample of the fluid was collected into PBS plus antibiotics. Statistically representative samples of ovarian fluid were also collected, according to the tables presented by Simon & Schill (1984).

Isolation procedures

Viscera samples from asymptomatic and diseased fish were processed in the same way, according to the

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TABLE 1 Cases of diseased rainbow trout from which viruses were isolated between January 1983 and December 1987

Date	Case No.	Site	Sample	No.	Cells
28/6/85	F33/85	Waterval	Fry	55	RTG2
28/6/85	F33/86(A)	Waterval	Fry	50	RTG2/BF2
28/6/85	F33/85(D)	Waterval	Fry	50	RTG2/BF2
28/8/85	F100/86	Machado R.T.	F	10	RTG2
3/9/86	F110/86	Mondi	S	95	RTG2/FHM
3/9/86	F112/86	Mondi	Fry	5	BF2/FHM
3/9/86	F119/86	Kingfisher	Fry	25	RTG2/BF2
3/9/86	F122/86	Sperwinger	Fry	25	RTG2/FHM
3/9/86	F125/86	E. Brewer	Fry	30	BF2/FHM
3/9/86	F126/86	E. Brewer	Fry	20	BF2/FHM
3/9/86	F127/86	E. Brewer	Fry	150	BF2/FHM
3/9/86	F130/86	Fisantekraal	Fry	15	RTG2/BF2
24/9/86	F135/86	Fisantekraal	Fry	15	RTG2/BF2

S = Slaughter fish and fish under 1 year
F = Fingerlings
Machado R. T. = Machado rainbow trout

TABLE 2 Successful virus isolation from viscera and ovarian fluid samples from asymptomatic fish

Date	Site	Age	Sample	No.	Cells
16/7/86	Mondi	S	V	120	RTG2/BF2
17/7/86	TPA Lydenburg	Fry	W	140	RTG2/BF2
24/7/86	Aquacultura	B.S.	O.F.	165	RTG2/BF2

S = Slaughter fish and fish under 1 year
B.S. = Brood stock
O.F. = Ovarian fluid
V = Viscera (liver, spleen & kidney)
W = Whole fish
TPA = Transvaal Provincial Administration

TABLE 3 Successful virus isolation from trout ova

Date	Case No.	Source	No.	Cells
2/11/85	F56/85	USA	200	RTG2/BF2

method described by Hill (1976) and Amos (1985). The samples were homogenized and diluted 1:10 with Eagle's balanced salt solution (BSS) (Hill, 1976). Bacterial contamination was removed by filtration through a 0,45 μm membrane filter. The filtered samples were inoculated in duplicate onto 95 % confluent cell sheets according to the methods reviewed by Hill (1976).

Samples of ova were pooled into 10 ova per pool and were disrupted by squeezing with sterile forceps. The samples were then diluted 1:10, filtered and inoculated onto cells by the methods described above. Ovarian fluid samples were not homogenized or diluted (Hill, 1976) but were filtered and inoculated onto the cells by the methods described above.

All of the inoculated cells were incubated at 10 °C and observed daily for the development of cytopathic effects (CPE). All cultures showing positive CPE were passaged to exclude toxicity of the sample (Hill, 1976). If the CPE persisted after passaging, it was assumed that a virus had been isolated. If no CPE were seen after 7 day's incubation, all samples were removed and re-inoculated onto fresh cells. If no CPE were seen after an additional 7 days the samples were discarded as negative.

Virus identification

Antiserum against VHS and the VR299, Ab and Sp serotypes of IPN were supplied by Dr de Kinkelin of France. Isolated viruses were identified by the serum neutralization test (Hill, 1976; Lientz & Springer, 1973), the indirect immunofluorescent antibody technique (Hill, 1976; Piper, Nicholson & Dunn, 1973) and the immunostaphylococcus-protein-A test (ISPA) (Bragg & Combrink, 1987a). In the ISPA test, cell cultures were inoculated with the isolated viruses and incubated at 10 °C until CPE were observed. The cells were then fixed with cold (−20 °C) acetone, and samples of virus-specific antibody sensitized *Staphylococcus aureus* Cowan strain A (Bragg & Combrink 1987a) were added to the fixed cell sheet. The cells were then washed 3 times with PBS and strained with May-Grunwald-Giemsa. The cells were observed and the average number of attached bacteria per cell calculated according to the methods described by Bragg & Combrink (1987a). The serological similarity between the South African isolates and the VR299, West Buxton, Ab and Sp serotypes of IPN was determined by Dr Hill of the Fish Disease Laboratory, Weymouth, England.

RESULTS

Cell culture

Samples of the RTG2, BF2 and FHM cell lines were obtained and successfully reconstituted, passaged and propagated. The average incubation time for the cells to reach confluency was calculated from laboratory records. The average incubation periods for the RTG2, BF2 and FHM cell lines were found to be 147,73 h, 100,17 h and 87,81 h respectively.

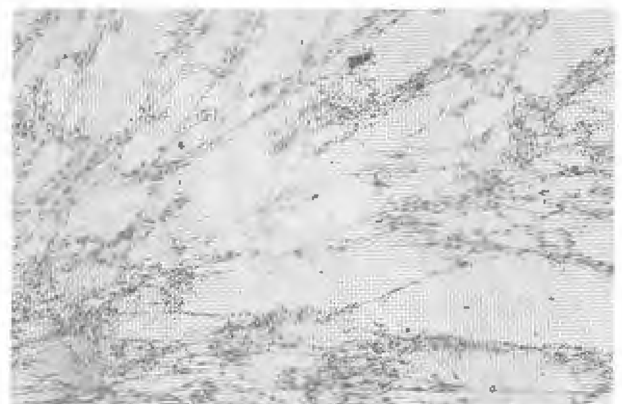

FIG. 1 Cytopathic effects caused by the virus isolated from case No. F33/85 on RTG2 cells

TABLE 4 Results of the serum neutralization test, IFAT and direct ISPA tests carried out on isolated viruses

Isolate	Antiserum	Neuts	IFAT	Direct ISPA: average No. bact/cell	
				Test	Control
F33/85	Anti IPN VR299	C	Pos	5,643	0,325
	Anti IPN Ab	P	Pos	2,651	0,342
	Anti IPN Sp	P	Pos	3,754	0,250
	Anti VHS	N	Neg	0,255	0,307
F33/85 (A)	Anti IPN VR299	C	Pos	6,652	0,462
	Anti VHS	N.D.	N.D.	N.D	N.D
F33/85 (D)	Anti IPN VR299	C	Pos	4,869	0,300
	Anti VHS	N.D	N.D	N.D	N.D
F100/85	Anti IPN VR299	C	Pos	5,723	0,405
	Anti VHS	N	Neg	0,355	0,264
F110/85	Anti IPN VR299	C	Pos	7,452	0,364
	Anti IPN Ab	P	Pos	2,753	0,265
	Anti IPN Sp	P	Pos	4,021	0,283
	Anti VHS	N	Neg	0,388	0,286
F112/86	Anti IPN VR299	C	Pos	5,710	0,355
	Anti VHS	N.D	N.D	0,324	0,211
F119/86	Anti IPN VR299	C	N.D.	5,324	0,332
	Anti VHS	N.D.	N.D.	0,197	0,265
F122/86	Anti IPN VR299	C	Pos	5,302	0,256
	Anti VHS	N.D.	N.D.	N.D.	N.D.
F125/86	Anti IPN VR299	C	Pos	5,610	0,280
	Anti VHS	N.D.	N.D.	0,321	0,275
F126/86	Anti IPN VR299	C	N.D.	N.D.	N.D.
	Anti VHS	N.D.	N.D.	N.D.	N.D.
F127/86	Anti IPN VR299	C	N.D.	N.D.	N.D.
	Anti VHS	N	N.D.	N.D.	N.D.
F130/86	Anti IPN VR299	C	Pos	4,679	0,217
	Anti VHS	N	Neg	N.D.	N.D.
F135/86	Anti IPN VR299	C	Pos	N.D.	N.D.
	Anti VHS	N	Neg	N.D.	N.D.
Fisantekraal (9/6/86)	Anti IPN VR299	C	Pos	6,847	0,329
	Anti IPN Ab	P	Pos	2,649	0,254
	Anti IPN Sp	P	Pos	2,869	0,196
	Anti VHS	N	Neg	0,235	0,301
Mondi (16/7/86)	Anti IPN VR299	C	Pos	5,754	0,245
	Anti IPN Ab	P	Pos	2,769	0,345
	Anti IPN Sp	P	Pos	3,005	0,200
	Anti VHS	N	Neg	0,356	0,256
TPA Lydenburg (16/7/86)	Anti IPN VR299	C	Pos	5,732	0,245
	Anti IPN Ab	P	Pos	2,629	0,222
	Anti IPN Sp	P	Pos	3,035	0,363
	Anti VHS	N	Neg	0,374	0,337
Aquacultura (24/7/86)	Anti IPN VR299	C	Pos	5,376	0,210
	Anti IPN Ab	P	Pos	3,025	0,243
	Anti IPN Sp	P	Pos	3,567	0,233
	Anti VHS	N	Neg	0,255	0,312
F56/86 (ova)	Anti IPN VR299	C	Pos	5,376	0,327
	Anti IPN Ab	P	Pos	2,834	0,301
	Anti IPN Sp	P	Pos	3,555	0,203
	Anti VHS	N	Neg	0,234	0,332

C = Complete neutralization; P = Partial neutralization
N = No neutralization; Pos = positive; Neg = Negative
N.D. = Not done

Virus isolation

The results obtained from the examination of diseased rainbow trout are summarized in Table 1. The CPE caused by the virus isolated from all of the cases were similar and can be seen in Fig. 1. From Table 1 it can be seen that viruses were isolated from Case No's F33/85, F33/85(A), F33/85(D), F100/86, F110/86, F112/86, F119/86, F122/86, F125/86 to F127/88, F130/86 and F135/86. These isolates were from a total of 8 sites.

The results obtained from the examination of viscera and ovarian fluid from asymptomatic fish can be seen in Table 2. Viruses were isolated on 3 occasions, from ovarian fluid, from slaughter fish and from fry. The CPE caused by these viruses were similar to those produced by the viruses isolated from diseased fish.

The results of the virological examination of trout ova are shown in Table 3. A virus was isolated from 1 consignment of imported ova.

Virus identification

All the isolated viruses were identified by the serum neutralization test, indirect fluorescent antibody test and the immunostaphylococcus-protein-A test. The results of these tests are summarized in Table 4. Typical results obtained when the serum neutralization test was done on the virus isolated from F33/85 are shown in Fig. 2 & 3. The attached bacteria in the direct ISPA test are shown in Fig. 4, while Fig. 5 shows the uninfected control cells. The isolated viruses were identified as IPN by IFAT, and were found to be serologically similar to the VR299 serotype by the serum neutralization test and the direct ISPA test. This is particularly noticeable in the direct ISPA test where the average number of attached bacteria per cell infected with the isolate and reacted with Anti IPN VR299 sensitized *S. aureus* was 6,146. The average number of attached bacteria per infected cell was only 2,403 when Anti Sp-sensitized *S. aureus* was used. The average number of attached bacteria per infected cell

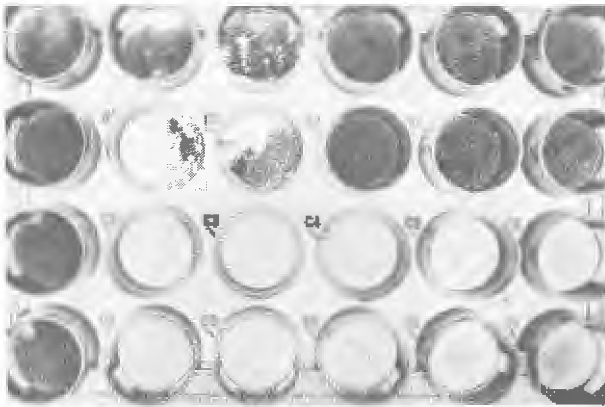

FIG. 2 Neutralization results when the virus isolated from case F33/85 was reacted with Anti IPN VR299. Wells A1–D1 were the cells controls. Wells A2–A6 and B2–B6 were the test wells, while wells C2–C6 and D2–D6 were the virus controls

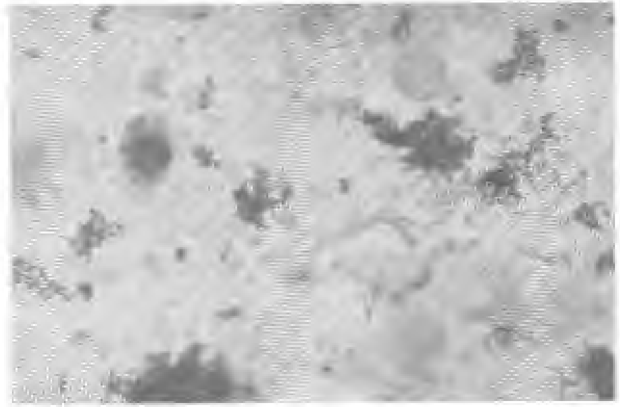

FIG. 4 Results obtained from the direct ISPA test. The cells were infected with the virus isolated from case F33/85 and the *S. aureus* used were sensitized with Anti IPN VR299. The attached bacteria can cleary be seen.

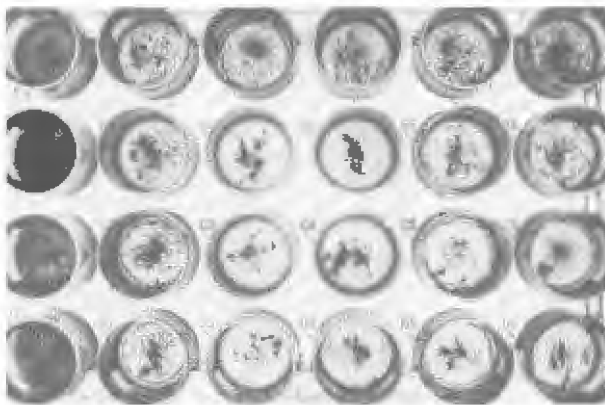

FIG. 3 No neutralization obtained when the virus isolated from case F33/85 was reacted with Anti VHS. Wells were the same as for Fig. 2.

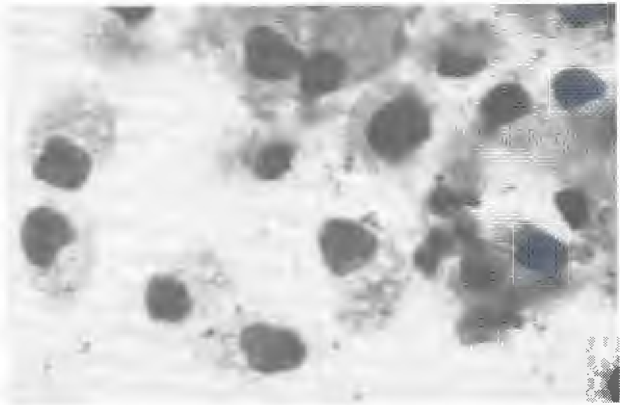

FIG. 5 Uninfected control RTG2 cells which were reacted with Anti IPN VR299 sensitized *S. aureus*. Some attached bacteria can be seen, but these are due to non-specific binding.

when Anti VHS-sentitized *S. aureus* was used was only 0,305. The serological similarity between the S.A. isolates and VR299 was confirmed by 50 % plaque neutralization titres (Hill, personal communication, 1985).

DISCUSSION

The first virus isolated from trout in South Africa was from fry submitted as Case F33/85 (Table 1). These fry were from ova imported from Denmark and the fact that a virus similar to the VR299 serotype of IPN was isolated led to a further investigation as this serotype has never been isolated in Europe. It was found that ova imported from the USA were hatched out in the same containers prior to the import of the Danish ova. Samples of fish from the American ova were collected [F33/85 (A)] and a similar virus was isolated from these fish. The next isolation of IPN in South Africa was from eyed trout ova imported from the USA (Case F56/85 in Table 3).

Virus was isolated for the first time during 1986 from samples collected from Fisantekraal in the Cape from asymptomatic fish about 8 months old. Viruses were then isolated from viscera of slaughter fish from a production site in Sabie and from fry at the TPA hatcheries at Lydenburg. IPN was also isolated from ovarian fluid collected at a farm near Belfast in the Transvaal. None of these sites reported high mortality rates and in all cases the viruses were isolated during routine monitoring. The only case from which mortalities were reported was Case No F100/86. IPN was isolated from fingerlings which came from Machado Rainbow Trout near Machadodorp but the mortalities were attributed to the presence of *Aeromonas salmonicida* in the fish (Bragg, unpublished data, 1986).

The isolation of IPN caused much concern and a survey of all fingerling producers was undertaken and samples were collected on 3 September 1985. Viruses similar to the VR299 serotype of IPN were isolated from 4 of the sites tested, 1 of which had tested positive before the survey. The other 3 sites had not been tested before. All the sites, except the provincial hatcheries at Lydenburg, had a history of recent imports of trout ova from a site in the USA. The hatchery at Lydenburg is, however, in close proximity to a number of the infected sites and it is possible that the virus was introduced onto this site via some vector.

As can be seen from Table 1, 2 & 3, no viruses were isolated from any site tested during 1987. A possible reason for this is that the virus was not capable of surviving the elevated water temperatures and high levels of ultraviolet radiation during the summer.

Apart from the viruses isolated from 13 cases during this period, an additional 123 cases, involving 2 599 fish from 34 different sites were examined and found to be free of IPN, IHN, VHS and herpesvirus salmonis. Mortalities in these cases could be attributed to other causes such as streptococcosis or enteric redmouth. A total of 6 968 viscera and 2 727 ovarian fluid samples from 23 sites were also tested and found to be free of IPN, IHN, VHS and herpesvirus salmonis. Apart from the single consignment of imported trout ova from which a virus

was isolated, an additional 5 000 ova from England, Denmark, USA and South Africa were tested and found to be free of IPN, IHN, VHS and herpesvirus salmonis.

It must be noted that all the viruses isolated were identified as being serologically similar to the VR299 serotype of IPN. None of the isolated viruses were identified as IHN, VHS or herpesvirus salmonis.

CONCLUSION

A virus serologically related to the VR299 serotype of IPN was isolated from 8 sites in the Transvaal and from 1 site in the Cape. The viruses were isolated from fry, fingerlings, slaughter fish, ovarian fluid and ova. High mortalities were not reported on any of the sites except 1 from which *A. salmonicida* was isolated.

All the sites except one, which is in close proximity to infected sites, had a recent history of imports from one site in the USA. A virus was also isolated from ova imported from this site. These facts, plus the fact that the virus was serologically similar to a North American serotype leads to the suggestion that the virus was introduced into South Africa via trout ova imported from the USA.

IHN, VHS and herpesvirus salmonis was not isolated from any of the samples examined during this time.

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