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Isolation And Identification Of InfectiousPancreatic Necrosis Virus From Rainbow Trout (salmo Gairdneri Richardson) In South Africa

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Fig 11. CPE caused by IHN virus on A) RTG2 cells, B) BF2 cells and C & D) FHM cells.

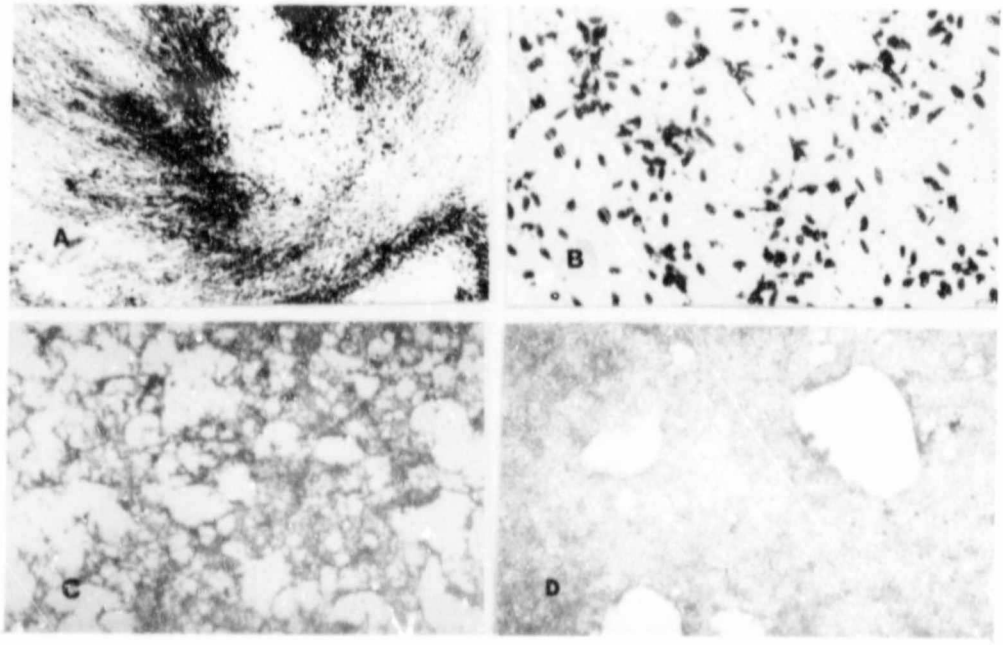
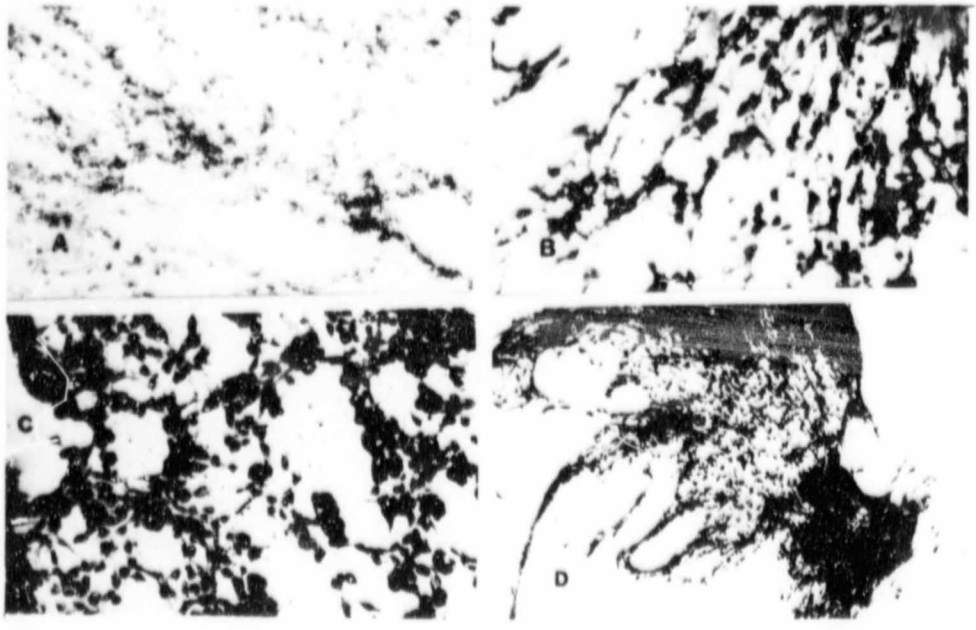


Fig 12. CPE caused by A) VHS virus on RTG2 cells, B) VHS virus on BF2 cells, C) VHS virus on FHM cells and D) herpesvirus salmonis on RTG2 cells.



The ability of the different viruses stored under different conditions to produce CPE after being reconstituted and inoculated onto cells by the methods described previously was determined and recorded in Table 5 - 8.

Table 5. Survival of viruses, as seen by the ability to produce CPE after storage in 50% glycerol

Virus	Number Stored	Numbe recor stitu	Positive	Negative	Survival (%)
IPN VR299	85	39	36	3	92,3
IPN Ab	72	33	24	9	72,7
IPN Sp	76	26	19	7	73,3
IHN	4	4	0	4	-
VHS	3	3	0	3	-
Herpesvirus salmonis	4	4	0	4	-

Table 6. Survival of viruses, as seen by the ability to produce CPE after storage by freezing at -20C.

Virus	Number Stored	Number recon-stituted	Positive	Negative	Survival (%)
IPN VR299	37	19	16	3	84,2
IPN Ab	42	16	14	2	87,5
IPN Sp	49	26	19	7	73.0
IHN	82	34	32	2	94,1
VHS	74	30	27	3	90,0
Herpesvirus salmonis	15	5	4	1	80,0

Table 7. Survival of viruses, as seen by the ability to produce CPE after storage by freezing in liquid nitrogen.

Virus	Number Stored	Number recon-stituted	Positive	Negative	Survival (%)
IPN VR299	32	17	17	0	100
IPN Ab	10	6	6	0	100
IPN Sp	12	7	7	0	100
IHN	24	13	13	0	100
VHS	22	13	11	2	83,3
Herpesvirus salmonis	7	2	2	0	100

Table 8. Survival of viruses, as seen by [illegible]ty to produce CPE after storage by freeze drying.

Virus	Number Stored	Number recon-stituted	Positive	Negative	Survival (%)
IPN VR299	5	3	3	0	100,0
IPN Ab	5	3	3	0	100,0
IPN Sp	5	3	3	0	100,0
IHN	5	3	3	0	100,0
VHS	5	3	3	0	100,0
Herpesvirus salmonis	5	2	2	0	100,0

4.3. Virus titrations

The titres of the viruses were calculated by the 50% tissue culture incidence level (TCID 50). All virus titrations were done on 24 well plates as described above. An example of the calculation of the titre of a stock sample of herpesvirus salmonis is presented below. The calculations are according to those of Reed & Muench (1938) and Luria et al (1978).

Example of the methods for the calculation of the titres of fish viruses.

Virus	Dilution	Number positive CPE	Number negative CPE	Total[1] number positive CPE	Total[2] number negative CPE	Percent mortality
Herpes-virus salmonis	0	4	0	18	0	100
	-2	4	0	14	0	100
	-4	4	0	10	0	100
	-5	3	1	6	1	85
	-6	3	1	3	2	60
	-7	0	4	0	6	0

1 = Total number of positive CPE as added from the highest dilution to the lowest dilution.

2 = Total number of negative CPE as added from the lowest dilution to the highest dilution.

If 50% CPE was obtained with a particular dilution, this dilution is used to calculate the TCID 50/ML. If, as is the case above, the 50% CPE falls between two dilutions, the proportional distance between these two dilutions must be calculated according to the methods set out below:

$$\frac{50\% - \%\ \text{CPE AT DILUTION BELOW}}{\%\ \text{CPE AT DILUTION ABOVE} - \%\ \text{CPE AT DILUTION BELOW}}$$

$$= \frac{50 - 0}{60 - 0} = \frac{50}{60} = 0{,}83$$

The TCID 50 was calculated as follows:

Log of the lower dilution minus the proportional distance times the log of the dilution factor.

$$\text{Log of the lower dilution} - 0{,}83 \times \log \text{dilution factor} = -6 - 0{,}83 = -6{,}83$$

0,1 ml of a $10^{-6,83}$ dilution = $1.5 \times 10^{-8} = 1$ TCID 50

Titre in TCID 50 per ml = $1/1.5 \times 10^{-8} = 6.66 \times 10^{7}$

The results of the titrations of a sample of the VR299, Ab and Sp serotypes of IPN virus, IHN virus, VHS virus and herpesvirus salmonis were recorded (Table 9). These results are from 10 repetitions on the same virus sample.

Table 9. Titres of a sample of each of the reference viruses.

Virus	TCID 50
IPN VR299	4.065×10^{6}
IPN Ab	1.747×10^{7}
IPN Sp	5.741×10^{6}
IHN	4.167×10^{6}
VHS	4.706×10^{5}
Herpesvirus salmonis	4.600×10^{7}

4.4. Plaque assay

The ability of the different serotypes of IPN virus to form plaques on the RTG2 cells were investigated as described. This technique was carried out a number of times and the results recorded (Table 10 & Fig 13).

Table 10. Results obtained from the plaque assay with the serotypes of IPN virus on RTG2 cells.

Serotype	Number of attempts	Number Successful	Number Unsuccessful	Success rate (%)
IPN VR299	6	3	3	50,0
IPN Ab	4	3	1	75,0
IPN Sp	6	4	2	66,6

The plaques were counted and used to determine the titre of the viruses in pfu/ml (Table 11).

Table 11. Titres of the viruses expressed in pfu/ml for the different serotypes of IPN virus. Results for IPN VR299 virus are from Fig 13.

Virus	Dilution	Volume (ml)	Number of Plaques	Titre/ dilution	Titre
IPN VR299	10^{-2}	0,5	34	6.8×10^{3}	
	10^{-3}	0,5	8	$1,6 \times 10^{4}$	
	10^{-4}	0,5	3	2×10^{4}	$1,42 \times 10^{4}$
IPN Ab	10^{-1}	0,5	17	3.4×10^{2}	
	10^{-2}	0,5	10	2×10^{3}	
	10^{-3}	0,5	4	8×10^{3}	3.45×10^{3}
IPN Sp	10^{-2}	0,5	35	7×10^{3}	
	10^{-3}	0,5	18	3.6×10^{4}	
	10^{-4}	0,5	8	1.6×10^{5}	6.77×10^{4}

Fig 13. Plaques produced by IPN VR299 on RTG2 cells. Well no. 1 is a negative control. Wells 2 - 6 are increasing 10-fold dilutions from 10^{-2} to 10^{-6} of IPN VR299 virus. Plaques can be seen in wells no. 2 - 4.

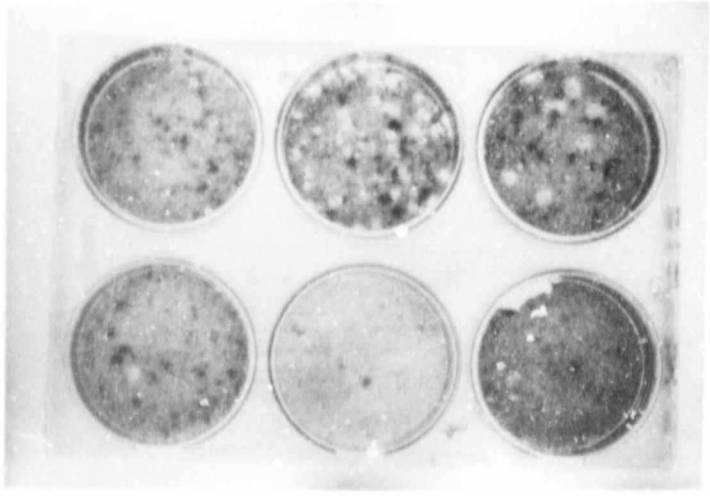
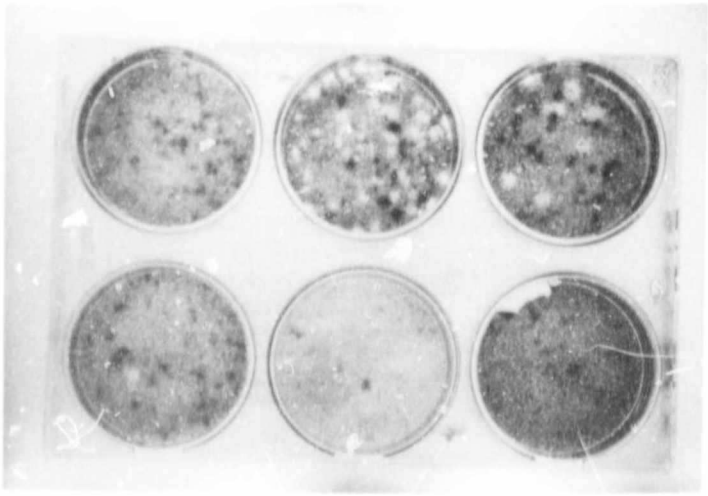


Table 11. Titres of the viruses expressed in pfu/ for the different serotypes of IPN virus. Results for IPN \ 299 virus are from Fig 13.

Virus	Dilution	Volume (ml)	Number of Plaques	Titre/ dilution	Titre
IPN VR299	10^{-2}	0,5	34	$6,8 \times 10^{3}$	
	10^{-3}	0,5	8	$1,6 \times 10^{4}$	
	10^{-4}	0,5	3	2×10^{4}	$1,42 \times 10^{4}$
IPN Ab	10^{-1}	0,5	17	3.4×10^{2}	
	10^{-2}	0,5	10	2×10^{3}	
	10^{-3}	0,5	4	8×10^{3}	3.45×10^{3}
IPN Sp	10^{-2}	0,5	35	7×10^{3}	
	10^{-3}	0,5	18	3.6×10^{4}	
	10^{-4}	0,5	8	1.6×10^{5}	6.77×10^{4}

Fig 13. Plaques produced by IPN VR299 on RTG2 cells. Well no. 1 is a negative control. Wells 2 - 6 are increasing 10-fold dilutions from 10^{-2} to 10^{-6} of IPN VR299 virus. Plaques can be seen in wells no. 2 - 4.



4.5. Virus concentration and electron microscopy

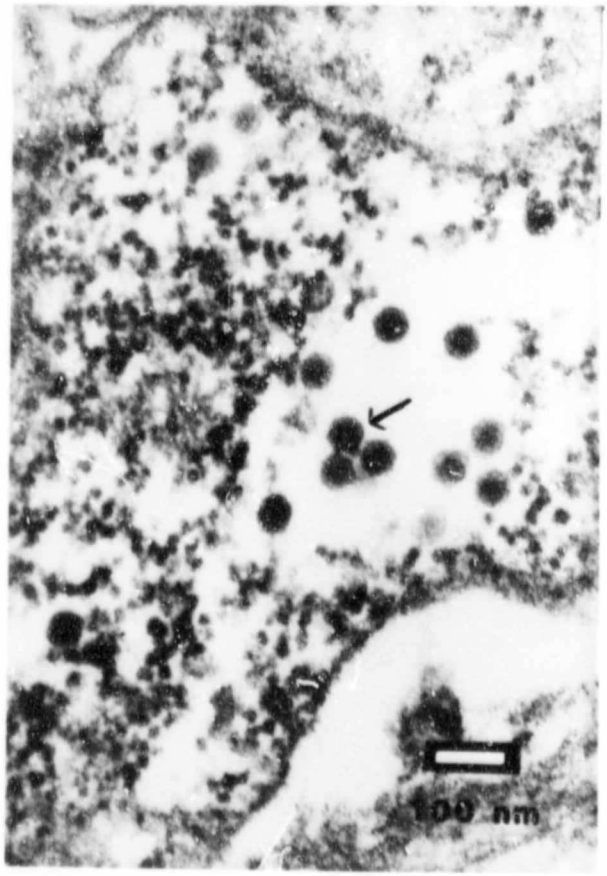
Samples of the VR299, Ab and Sp serotypes of IPN virus were concentrated by ultra-centrifugation and the pellets resuspended as described previously. The viruses were negatively stained according to the methods described previously and examined in the electron microscope (Fig 14). A number of empty capsules can be seen (Fig 14 arrowed) in the negatively stained preparation of concentrated virus particles.

Samples of cell cultures infected with these viruses were prepared according to the methods set out above, sectioned and studied in the E.M. (Fig 15).

Fig. 14. Electron micrographs of concentrated IPN VR299 virus from RTG2 cells showing CPE which have been stained with 2% phosphotungstic acid. Empty virus particles can be seen (arrowed)



Fig. 15. Electron micrograph of IPN VR299 (arrowed) in ultrathin section of infected RTG2 cells.



4.6. Serological identification of IPN virus.

4.6.1. Antisera

Antibodies against IPN VR299 virus were raised in rabbits. according to the methods discussed previously. The titre of the virus used for the inoculation of the rabbits was calculated according to the methods of Reed & Muench (1938) and found to be $3,1623 \times 10^{-8}$ TCID 50/ML. After the last inoculation the rabbits were bled and the specificity of the antibodies tested according to the methods discussed in the Methods and Materials section.

Rabbit anti IPN VR299 serum diluted to 1/1000 was capable of neutralizing all the viruses in a 10^{-4} dilution of the stock IPN VR299 sample (titre = $3,1623 \times 10^{-8}$ TCID 50/ML). Samples of the antiserum were stored in liquid nitrogen. Antibodies against the VR299 serotype of IPN virus were raised only once in rabbits. No neutralizing activity was found when samples of rabbit raised anti IPN VR299 was mixed with VHS virus or IHN virus, thus indicating that the antibodies raised in the rabbits were specific for IPN virus.

4.6.2. Serological tests

4.6.2.1. Indirect immunofluorescent antibody test.

The indirect immunofluorescent antibody test was carried out according to the techniques discussed in the Methods and Materials section and the results recorded (Table 12, Fig 16). The indirect immunofluorescent antibody test (IFAT) was carried out on the different serotypes of IPN virus and VHS virus. Photographic evidence of this technique carried out on non-infected cells is not presented as these were entirely dark and not possible to photograph.

Table 12. Results of the indirect immunofluorescent antibody tests carried out on cells infected with IPN virus or VHS virus.

Virus used to infect cells	Antiserum	No. of test	Number Successful	Number unsuccessful
IPN VR299	IPN VR299	13	11	2
IPN Ab	IPN Ab	9	8	1
IPN Sp	IPN Sp	8	7	1
VHS	VHS	15	15	0
IPN VR299	Poly.[2] IPN	4	4	0
IPN Ab	Poly. IPN	2	2	0
IPN Sp	Poly. IPN	3	3	0
IPN VR299	IPN Ab	3	3	0
IPN VR299	IPN Sp	3	2	1
IPN Ab	IPN VR299	4	2	2
IPN Ab	IPN Sp	3	2	1
IPN Sp	IPN VR299	4	3	1
IPN Sp	IPN Ab	4	2	2
VHS	poly. IPN	5	0	5
IPN VR299	VHS	5	0	5
IPN Ab	VHS	2	0	2
IPN Sp	VHS	2	0	2
Control cells[1]	IPN VR299	5	0	5
Control cells	IPN Ab	5	0	5
Control cells	IPN Sp	5	0	5
Control cells	VHS	8	0	8

1 = Uninfected cells used as negative controls
2 = Group polyvalent anti IPN serum

4.6.2.2. Immunoperoxidase test.

The indirect immunoperoxidase test was done according to the methods described earlier. A positive result was recorded when a brown precipitate was observed on the cells (Fig 17). The indirect immunoperoxidase assay was only carried out twice on RTG2 cells infected with IPN virus and twice on RTG2 cells infected with VHS virus. There are thus not sufficient repetitions to draw any meaningful conclusions on the use of this technique for the serological identification of these viruses. It should however be noted that positive results were obtained in all four tests when using homologous virus and antisera.

Fig 16. Positive immunofluorescence on RTG2 cells infected with IPN VR299 virus and stained by the indirect immunofluorescent antibody technique.

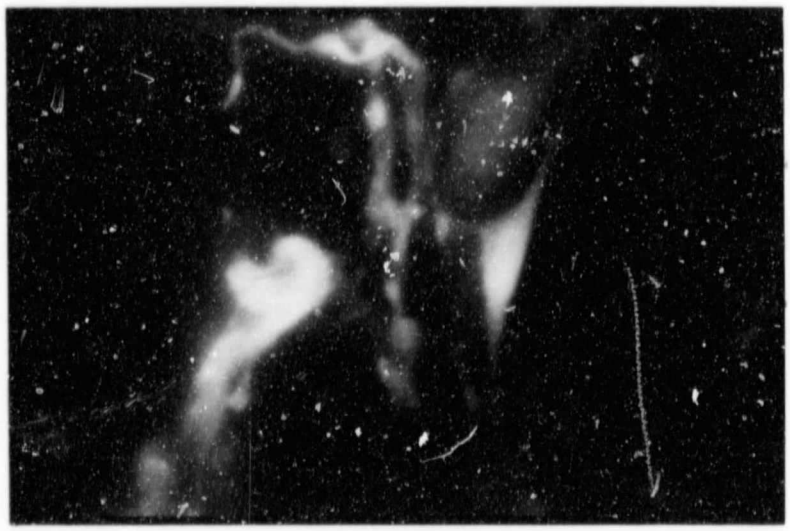
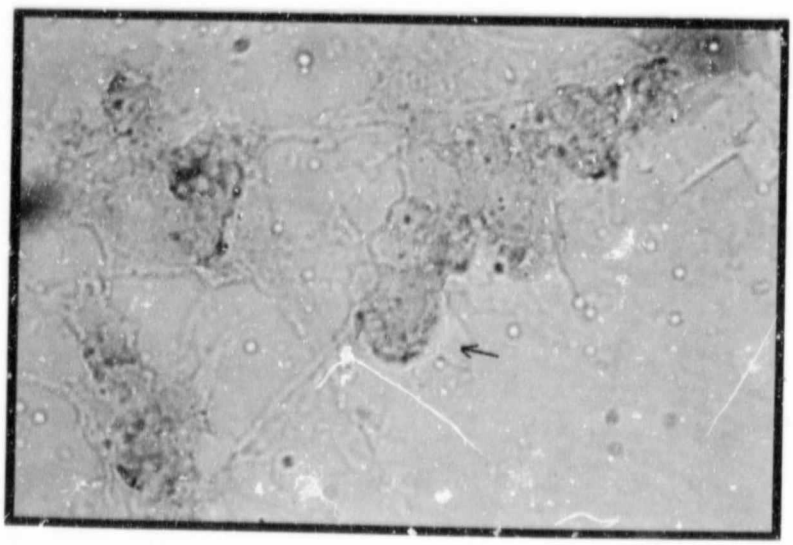


Fig 17. Brown precipitate (arrowed) indicating a positive result when the indirect immunoperoxidase assay was done on RTG2 cells infected with IPN VR229 virus.



4.6.2.3. Serum neutralization test.

The serum neutralization test was carried out successfully a number of times. Serum neutralizations were done on the RTG2, BF2 and FHM cells and the results recorded (Table 13). Typical neutralization results obtained when IPN VR299 virus was mixed with anti IPN VR299 and inoculated onto RTG2 cells on a 24 well plate can be seen in Fig 18. Fig 19 represents a typical negative result which was obtained if IPN VR299 virus was first mixed with anti VHS and then inoculated onto the cells. The vertical row of wells on the left of the plate were negative controls. These four wells were inoculated with MEM. The top two horizontal rows of wells, excluding the first well of each row, were inoculated with an increasing dilution of IPN VR299 virus which had been mixed with equal volumes of anti IPN VR299 or anti VHS. The bottom two horizontal rows of wells, excluding the first well of each row, were inoculated with a dilution of IPN VR299 virus, corresponding to the dilution used to inoculate the wells above, which had been mixed with equal volumes of FCS.

The negative control wells were undamaged in both cases while the cells inoculated with the diluted virus which had been mixed with FCS were completely destroyed in both (Fig 18 & 19). The wells which were inoculated with IPN VR299 virus which had been mixed with anti VHS were also destroyed, thus indicating that the virus had not been neutralized (Fig 19). The last three wells which were inoculated with dilutions of IPN VR299 virus which had been mixed with anti IPN VR299 were undamaged, thus indicating that the virus had been neutralized. Partial neutralization was found when the sample of IPN VR299 virus was diluted to 10^{-1} or 10^{-2} and mixed with anti IPN VR299 as can be seen from the second and third wells in the top two vertical rows in Fig 18.

Table 13. Results of the serum neutralization test on different cell lines.

Virus	Antiserum	Neut. index greater than 1	Neut. index less than 1	Average Neut. index
IPN VR299	IPN VR299	8	0	>6
IPN Ab	IPN Ab	6	0	>6
IPN Sp	IPN Sp	8	0	>6
VHS	VHS	11	0	>6
IPN VR299	Polyvalent [1]	4	0	>6
IPN Ab	Polyvalent	2	0	>6
IPN Sp	Polyvalent	4	0	>6
IPN VR299	IPN Sp	3	1	2.50
IPN VR299	IPN Ab	2	2	1.80
IPN Sp	IPN VR299	4	0	3.70
IPN Sp	IPN Ab	2	2	2.10
IPN Ab	IPN VR299	2	1	2.00
IPN Ab	IPN Sp	2	1	1.95
IPN VR299	VHS	0	4	<1
VHS	Polyvalent	0	4	<1

1 = Group polyvalent antiserum against the serotypes of IPN

Fig 18. Positive neutralization obtained when an increasing dilution of IPN VR299 virus was mixed with anti IPN VR299.

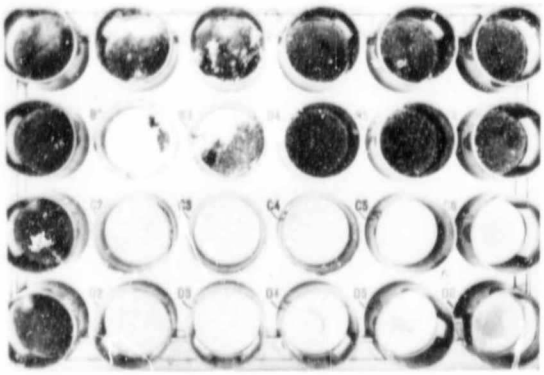


Fig 19. No neutralization obtained when diluted samples of IPN VR299 virus were mixed with anti VHS.



4.6.2.4. Direct immunostaphylococcus-protein-A test.

Samples of S. aureus Cowan strain A were successfully reconstituted and found to be pure cultures according to the methods described previously. The formalin inactivation of the bacteria was successful, as can be seen by the absence of growth on samples plated onto BTA plates. Samples of bacteria which had not been formalin inactivated did grow on the BTA plates under the same conditions. The successful binding of viral specific antibodies to the formalized bacteria was tested by the fluorescent antibody technique or the immunoperoxidase assay according to the methods described in the Methods and Materials section. Fluorescence, or a brown precipitate was seen when sensitized S. aureus bacteria were reacted with FITC or peroxidase labelled anti-rabbit (Fig 20 & 21). No fluorescence or brown precipitate was seen when unsensitized S. aureus was reacted with either FITC or peroxidase labelled anti-rabbit. No fluorescence or brown precipitate was found when sensitized and unsensitized S. aureus were reacted with FITC or peroxidase labelled goat anti-mouse.

Fig 20. Fluorescence seen on S. aureus Cowan strain A cells which were sensitized with rabbit raised antibodies and stained with FITC labelled anti-rabbit.

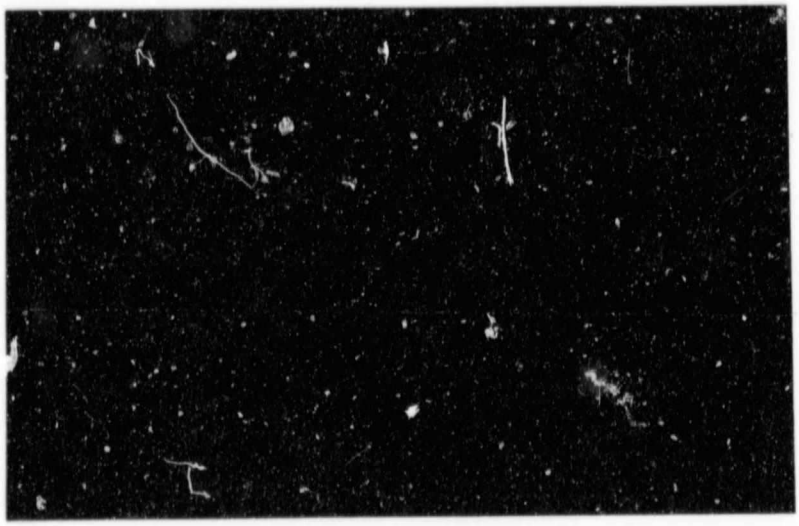
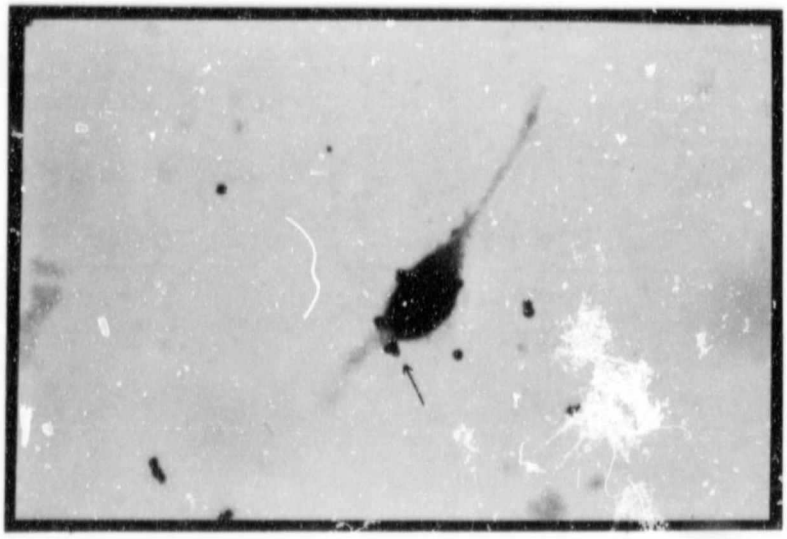


Fig 21. Results obtained when S. aureus Cowan strain A bacteria which were sensitized with rabbit raised antibodies, were overlayed with peroxidase labelled anti-rabbit and covered with 3'3 diaminobenzidine tetrachloride. The bacteria are a brownish colour as a result of the reaction between the peroxidase and the substrate.



The direct ISPA test was successfully carried out a number of times according to the methods discussed previously. The viral specific antibody sensitized bacteria were found to attach to the viral infected cells if the antibodies and virus were homologous (Fig 22). The number of attached bacteria were counted and these numbers were used to calculate the average number of attached bacteria per cell (Table 14). In the cases were the virus and antibodies were not homologous, or when uninfected cells were used, few bacteria were found to attach to the cells (Fig 23). These bacteria were also counted and recorded (Table 14).

Fig 22. Viral specific antibody sensitized S. aureus Cowan strain A (arrowed) attached to viral infected cells when the antibodies and virus were homologous.



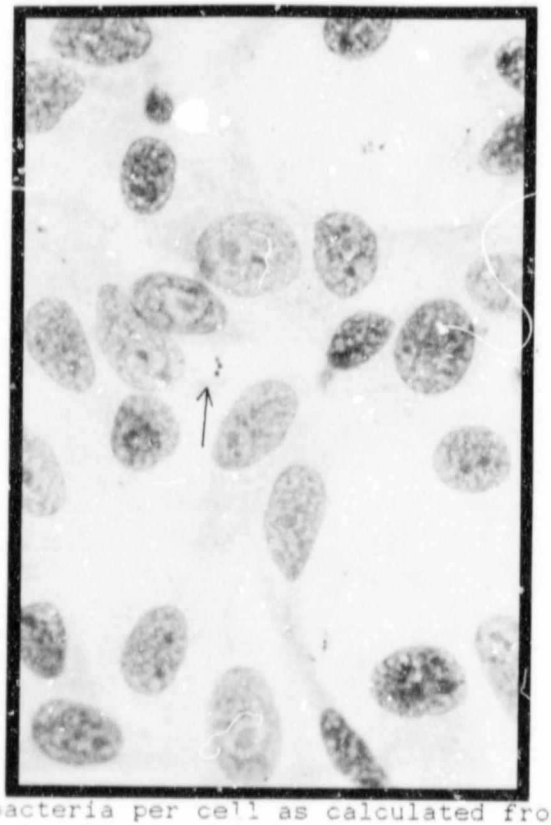


Fig 23. Viral specific antibody sensitized S. aureus Cowan strain A (arrowed) non-specifically bound to uninfected control cells. The number of attached bacteria is greatly reduced when compared to Fig 22

Table 14. Average number of bacteria per cell as calculated from the direct ISPA test. Control cells in this table were cells which were uninfected.

Virus used to infect cells	Antibodies used to label S. aureus	Number of tests	Average No of Bact./cell	
			Infected cells	Control cells
IPN VR299	Anti IPN VR299	5	3.7	0.3
IPN Ab	Anti IPN Ab	5	4.1	0.2
IPN Sp	Anti IPN Sp	5	5.8	0.5
IPN VR299	Anti Poly.[1] IPN	5	4.2	0.2
IPN Ab	Anti Poly. IPN	5	4.3	0.3
IPN Sp	Anti Poly. IPN	5	3.8	0.3
VHS	Anti VHS	5	2.3	0.1
IPN VR299	Anti IPN SP	5	2.7	0.2
IPN VR299	Anti IPN Ab	5	1.9	0.4
IPN Sp	Anti IPN VR299	5	3.1	0.2
IPN Sp	Anti IPN Ab	5	2.1	0.2
IPN Ab	Anti IPN VR299	5	1.8	0.3
IPN Ab	Anti IPN Sp	5	2.3	0.3
VHS	Anti IPN VR299	5	0.2	0.2
VHS	Anti Poly IPN	5	0.3	0.3
IPN VR299	Anti VHS	5	0.2	0.2

1 = Group polyvalent anti IPN serum.

4.6.2.5. Indirect ISPA test

The results of the stabilization of S. aureus Cowan strain A were similar to those obtained for the direct method. The stabilized S. aureus for the indirect ISPA test were successfully sensitized with goat anti-rabbit as described. The results of the indirect ISPA test are given in Table 15. It was found that the bacteria sensitized with anti-rabbit IgG attached to the cells if the rabbit raised viral specific antibodies used to overlay the cells corresponded with the virus which was used to infect the cells (Fig 24). However, some bacteria still attached, non-specifically to the negative control cells (Fig 25).

Fig 24. Attached bacteria (arrowed) on virus infected cells in a successful indirect ISPA test.

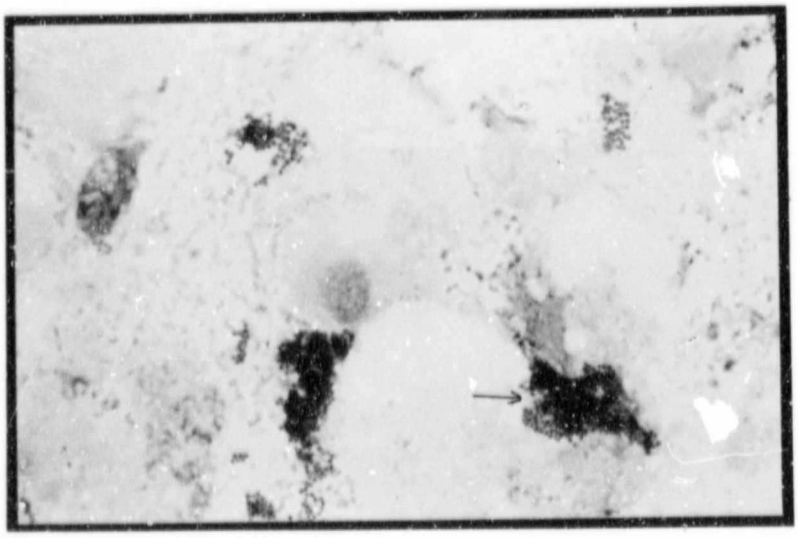


Fig 25. Some attached bacteria (arrowed) attached to negative control cells. The number of bacteria is greatly reduced when compared to Fig 24.

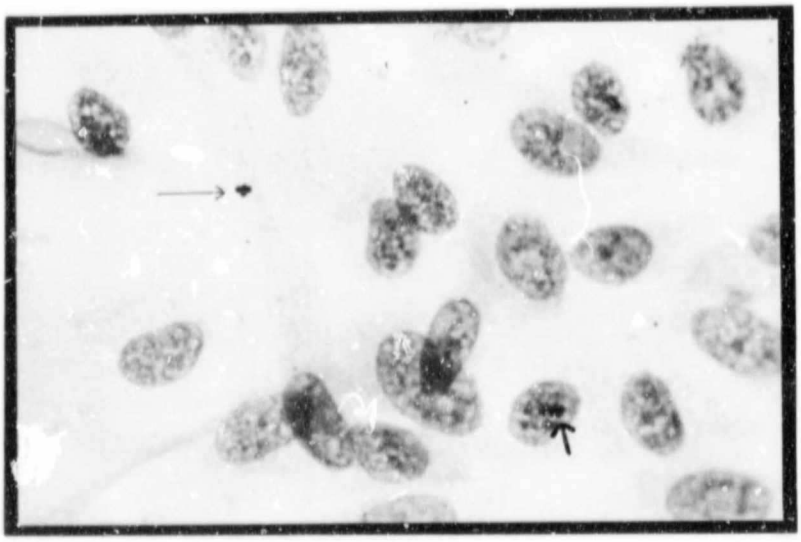


Table 15. The average number of bacteria per cell as calculated from the indirect ISPA test. The S. aureus for these tests were sensitized with goat anti-rabbit. Control cells in this table were uninfected cells.

Virus used to infect cells	Cells overlayed with rabbit raised:	Number of tests	Average No of Bact./cell	
			Infected	Control
IPN VR299	Anti IPN VR299	5	10.9	0.6
IPN Ab	Anti IPN Ab	5	2.7	0.5
IPN Sp	Anti IPN Sp	5	8.1	0.8
IPN VR299	Anti Poly. IPN	5	6.5	0.3
IPN Ab	Anti Poly. IPN	5	5.4	0.3
IPN Sp	Anti Poly. IPN	5	3.7	0.3
VHS	Anti VHS	5	4.9	0.7
VHS	Anti IPN VR299	5	0.7	0.6
VHS	Anti Poly IPN	5	0.3	0.4
IPN VR299	Anti VHS	5	0.4	0.4

4.6.2.6. Viral induced bacterial co-agglutination test. (VIBCA)

The viral induced bacterial co-agglutination (VIBCA) test was done a number of times (Table 16, Fig 26). Controls consisted of combinations of different viruses with unsensitized S. aureus and combinations of virus and S. aureus which had been sensitized with heterologous virus specific antibodies.

Fig 26. Results of the VIBCA test. A) Positive VIBCA, showing clumps of bacteria and B) negative controls showing an even distribution of bacteria.

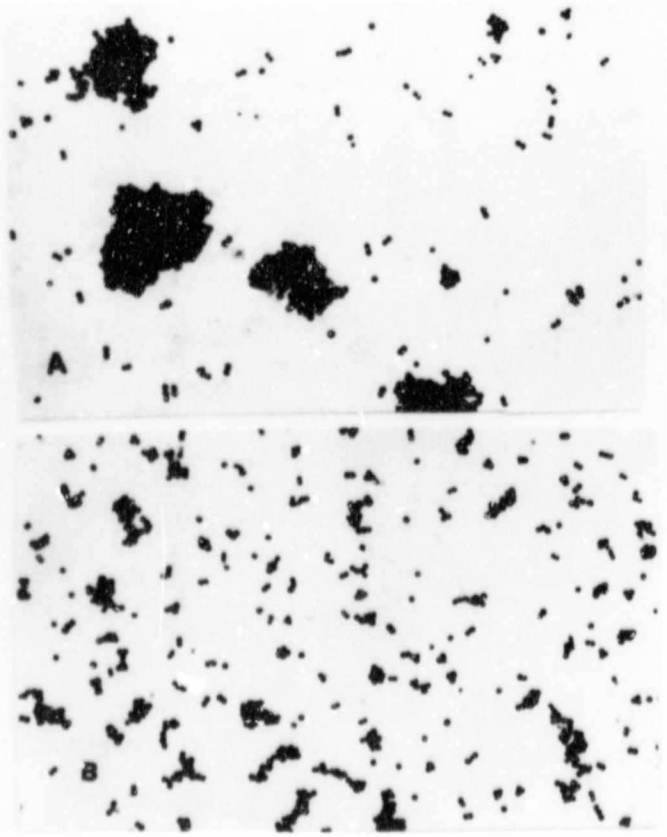


Table 16. Results of the VIBCA test with different combinations of antigens and sensitized S. aureus Cowan strain A bacteria.

Virus.	Sensitized S. aureus	No. pos	No. neg
IPN VR299	Anti IPN VR299	8	1
IPN Ab	Anti IPN Ab	4	0
IPN Sp	Anti IPN Sp	5	0
VHS	Anti VHS	10	0
IPN VR299	Anti polyvalent IPN	5	0
IPN Ab	Anti polyvalent IPN	5	0
IPN Sp	Anti polyvalent IPN	5	0
VHS	Anti IPN VR299	0	5
VHS	Anti polyvalent IPN	0	5
IPN VR299	Anti VHS	0	5
IPN Ab	Anti VHS	0	3
IPN Sp	Anti VHS	0	3
Medium	Anti IPN VR299	0	5
Medium	Anti IPN Ab	0	4
Medium	Anti IPN Sp	0	4
Medium	Anti polyvalent IPN	0	3
Medium	Anti VHS	0	3
IPN VR299	Unsensitized	0	2
IPN Ab	Unsensitized	0	2
IPN Sp	Unsensitized	0	2
VHS	Unsensitized	0	2

4.7. Comparison of different serological techniques.

The titre of the virus sample used for the comparison of the serological techniques was calculated as $3{,}125 \times 10^{7}$ TCID 50/ml by the methods described earlier. The virus could be identified by IFAT and the indirect ISPA tests when the sample had a titre of above 3.125×10^{2} TCID 50/ml. The direct ISPA and the serum neutralization test were successful if the sample had a titre of above 3.125×10^{1} TCID 50/ml. The VIBCA test was only successful if the sample had a titre above 3.125×10^{6} TCID 50/ml.

4.8. Virus isolation and identification from diseased fish in South Africa.

Samples of diseased fish were received from the field and virus isolations on all the samples were attempted. A total of 3 147 samples of fish from 137 cases were investigated from January 1983 to the end of August 1987. Viruses were isolated from 11 different cases (Table 17). A negative result was recorded if no CPE were found after two passages of the sample. If cell damage was obtained on the first passage but not on the second passage these samples were regarded as negative (Hill 1976a). A positive result was only recorded if a sample was positive on the first and second passage or on the second passage alone. All samples which showed positive results were serologically identified and the results recorded (Table 18). A complete list of all cases processed from January 1983 to the end of August 1987 is presented in Appendix 2.

All positive isolations produced CPE indistinguishable from those produced by IPN virus (Fig 27).

Table 17. Cases from which viruses were isolated from January 1983 to the end of August 1987.

Date	Case No.	Site	Age	Number of fish	Cells
28/6/85	F33/85	Waterval	Fry	155	RTG2/BF2
28/8/86	F100/86	Machado R.T.[1]	Finger[2]	10	RTG2
3/9/86	F110/86	Mondi Hatchery	Fry	95	RTG2/FHM
3/9/86	F112/86	Mondi Hatchery	Fry	5	BF2/FHM
3/9/86	F119/86	Kingfisher	Fry	25	RTG2/BF2
3/9/86	F122/86	Sperwing	Fry	25	RTG2/FHM
3/9/86	F125/86	Katrina's Rust	Fry	30	BF2/FHM
3/9/86	F126/86	Katrina's Rust	Fry	20	BF2/FHM
3/9/86	F127/86	Katrina's Rust	Fry	150	BF2/FHM
3/9/86	F130/86	Fisantekraal	Fry	15	RTG2/BF2
24/9/86	F135/86	Fisantekraal	Fry	15	RTG2/BF2

1 = Machado Rainbow Trout
2 = Fingerlings

Table 18. Results of the serum neutralization test, IFAT and the direct ISPA test carried out on the viruses isolated from diseased fish.

Case No	Antiserum	Neuts[1]	IFAT[2]	direct ISPA[3] average no bact/cell	
				Infected	Control
F33/85	Anti IPN VR299	C[4]	Pos[7]	5,6	0,3
	Anti IPN Ab	P[5]	Pos	2,7	0,3
	Anti IPN Sp	P	Pos	3,8	0,3
	Anti VHS	N[6]	Neg[8]	0,3	0,3
F33/85 (A)	Anti IPN VR299	C	Pos	6,7	0,5
	Anti VHS	N.D.	N.D.[9]	N.D.	N.D.
F33/85 (D)	Anti IPN VR299	C	Pos	4,2	0,4
	Anti VHS	N.D.	N.D.	N.D.	N.D.
F100/86	Anti IPN VR299	C	Pos	5,7	0,4
	Anti VHS	N	Neg	0,4	0,3
F110/86	Anti IPN VR299	C	Pos	7,5	0,7
	Anti IPN Ab	P	Pos	2,8	0,3
	Anti IPN Sp	P	Pos	4,0	0,3
	Anti VHS	N	Neg	0,4	0,3
F112/86	Anti IPN VR299	C	Pos	5,7	0,4
	Anti VHS	N.D.	N.D.	0,3	0,2
F119/86	Anti IPN VR299	C	N.D.	5,3	0,3
	Anti VHS	N.D.	N.D.	0,2	0,3
F122/86	Anti IPN VR299	C	Pos	5,3	0,3
	Anti VHS	N.D.	N.D.	N.D.	N.D.
F125/86	Anti IPN VR299	C	Pos	5,6	0,3
	Anti VHS	N.D.	N.D.	0,3	0,3
F126/86	Anti IPN VR299	C	N.D.	N.D	N.D.
	Anti VHS	N.D.	N.D.	N.D.	N.D.
F122/86	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	4,7	0,2
	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.

1 = Neutralization: 2 = Indirect immunofluorescent antibody test
3 = Immunostaphylococcus-Protein-A test
4 = complete neutralization:
5 = Partial neutralization: neutralization index greater than 2 but less than 3.
6 = no neutralization: neutralization index less than 1.
7 = Positive immunofluorescence 8 = Negative immunofluorescence
9 = Not done.

Fig 27. CPE on RTG2 cells produced by the viruses isolated from the cases presented in Table 17.



4.9. Isolation and identification of viruses from viscera samples of asymptomatic fish.

Samples of rainbow trout were collected from farms at six monthly intervals and processed according to the methods discussed in the Methods and Materials section. A total of 7 133 samples of asymptomatic fish, consisting of 1 905 fry, 1 385 fingerlings and 3 843 slaughter fish were collected and tested (Appendix 3). Negative results were listed if no CPE were seen after two passages. Positive results were listed if any well showed CPE after the first and second passages (Table 19). If CPE were only found on the first passage and not the second passage, a negative result was recorded. All positive isolations after two passages were serologically identified (Table 20). The CPE caused by the viruses isolated from viscera of asymptomatic fish were similar to the CPE produced by the virus isolated from diseased fish which can be seen in Fig 27.

Fig 27. CPE on RTG2 cells produced by the viruses isolated from the cases presented in Table 17.



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Table 19. Viruses isolated from samples of asymptomatic fish.

Date	Site	Age	Sample	Number of fish	Cells
9/6/86	Fisantekraal	S[3]	V[1]	150	RTG2/BF2
16/7/86	La Rochelle	S	V	120	RTG2/BF2
17/7/86	TPA[4] Lydenburg	Fry	W[2]	140	RTG2/BF2

1 = Viscera (Liver, spleen & kidney)
2 = Whole fish (fish too small to eviscerate)
3 = Slaughter fish (and less than 1 year old)
4 = Transvaal Provincial Administration

Table 20. Results of the serum neutralization test, IFAT and the direct ISPA test done on the viruses isolated from viscera samples of asymptomatic fish.

Date	Site	Antiserum	Neuts[1]	IFAT[2]	direct ISPA[3] Average no. bact/cell	
					Test	Control
9/6/86	Fisantekraal	Anti IPN VR299	C	Pos	6,8	0,3
		Anti IPN Ab	P	Pos	2,6	0,3
		Anti IPN Sp	P	Pos	2,9	0,2
		Anti VHS	N	Neg	0,2	0,3
16/7/86	La Rochelle	Anti IPN VR299	C	Pos	5,8	0,2
		Anti IPN Ab	P	Pos	2,8	0,3
		Anti IPN Sp	P	Pos	3,0	0,2
		Anti VHS	N	Neg	0,4	0,3
16/7/86	TPA Lydenburg	Anti IPN VR299	C	Pos	5,7	0,2
		Anti IPN Ab	P	Pos	2,6	0,2
		Anti IPN Sp	P	Pos	3,0	0,4
		Anti VHS	N	Neg	0,4	0,3

1 = Neutralization
2 = Indirect immunofluorescent antibody test
3 = Immunostaphylococcus-Protein-A test

4.10. Isolation and identification of viruses from ovarian fluid samples from asymptomatic fish.

Samples of ovarian fluid were collected as part of a health certification scheme on some farms each year during the normal breeding operations on the sites. A total of 2 892 samples, from 13 sites were collected (Appendix 4) and only one site was found to be positive for IPN virus (Appendix 4). The virus which was isolated caused similar CPE on the cells to the virus isolated from diseased fish (Fig 27). This virus was serologically identified as IPN virus by IFAT (Table 21) and as the VR299 serotype by the serum neutralization test and the ISPA test (Table 21).

Table 21. Results of the serological identification of the virus isolated from ovarian fluid from asymptomatic fish.

Date	Site	Antiserum	Neut[1]	IFAT[2]	Direct ISPA[3] Average No. bact/cell	
					Test	Control
24/7/86	Aquacultura	Anti IPN VR299	C	Pos	5,4	0,2
		Anti IPN Ab	P	Pos	3,0	0,2
		Anti IPN Sp	P	Pos	3,6	0,2
		Anti VHS	N	Neg	0,3	0,3

1 = Serum neutralization
2 = Indirect fluorescent antibody technique
3 = Immunostaphylococcus-Protein-A test

4.11. Isolation and identification of viruses from eyed ova.

Samples of eyed ova were collected, either at Jan Smuts Airport or on farms, and the virus isolation procedures were attempted. A total of 5 200 ova from 22 consignments were examined (Appendix 5) and a virus was isolated from one consignment of ova imported from the USA on 2/11/85. The CPE produced by this virus was similar to the CPE produced by the virus isolated from diseased

fish (Fig 27). The virus was serologically identified as the VR299 serotype of IPN by the serum neutralization test and the direct ISPA test (Table 22).

Table 22. Results of the serological identification of the virus isolated from eyed trout ova.

Date	Case No	Antiserum	Neut[1]	IFAT[2]	Direct ISPA[3] Average No Bact/Cell	
					Test	Control
2/11/85	F56/86	Anti IPN VR299	C	Pos	5,4	0,3
		Anti IPN Ab	P	Pos	2,8	0,3
		Anti IPN Sp	P	Pos	3,6	0,2
		Anti VHS	N	Neg	0,2	0,3

1 = Serum neutralization test
2 = Indirect fluorescent antibody test
3 = Immunostaphylococcus-Protein-A test

4.12. Detection of antibodies in trout serum

Blood samples were successfully collected by the methods set out in the Methods and Materials section. Samples of the serum were used to do the virus neutralization test. A total of 215 serum samples, from 7 different sites were collected (Table 23). Viruses used in the virus neutralization tests were the VR299, Ab and Sp serotypes of IPN virus and VHS virus. Neutralizing antibodies against the VR299 serotype of IPN virus were detected in 23 of the 215 serum samples (Table 23).

Table 23. Serum samples collected for the detection of neutralizing antibodies.

Site/Case	Sample No.	Virus	Cells	Results
Mondi (Hatch)	1 - 7	VHS	RTG2/BF2	N[2]
Pleasantways	1 - 6	VHS	RTG2/BF2	N
S. Gifford	1 - 6	VHS	RTG2/BF2	N
Mondi (Hatch)	1 - 7	IPN VR299	RTG2/BF2	N
Pleasantways	1 - 6	IPN VR299	RTG2/BF2	N
S. Gifford	1 - 6	IPN VR299	RTG2/BF2	N
Waterval	A1	IPN VR299	RTG2/BF2	N
(F33/85)	A2	IPN VR299	RTG2/BF2	C[1]
"	A3 - A8	IPN VR299	RTG2/BF2	N
"	A9	IPN VR299	RTG2/BF2	C
"	A10	IPN VR299	RTG2/BF2	N
"	D1 - D5	IPN VR299	RTG2/BF2	C
"	D6 - D7	IPN VR299	RTG2/BF2	N
"	D8	IPN VR299	RTG2/BF2	C
"	D9 - D10	IPN VR299	RTG2/BF2	N
"	A1 - A10	VHS	RTG2/BF2	N
"	D1 - D10	VHS	RTG2/BF2	N
Waterval	1 - 6	IPN VR299	RTG2/BF2	N
Waterval	1 - 6	VHS	RTG2/BF2	N
Mondi (La Rochelle)	1	IPN VR299	RTG2/BF2	N
Mondi (La Rochelle)	2 - 8	IPN VR299	RTG2/BF2	C
Mondi (La Rochelle)	9	IPN VR299	RTG2/BF2	N
Mondi (La Rochelle)	10 - 14	IPN VR299	RTG2/BF2	C

1 = Complete neutralization
2 = No neutralization

5. DISCUSSION

5.1. Tissue culture

The establishment of the procedures for the passage and propagation of fish cell lines was the first priority of this project. To this end, samples of RTG2, BF2, FHM and BB cells were obtained from the ATCC. These particular cell lines were used firstly because of there commercial availability from the ATCC through various supply companies in this country (Flow Laboratories or Sterilab Services) and secondly because various authors state that these cell lines are the most sensitive to use for isolation of viruses from fish. Hill (1976a) states that the RGT2 and BF2 cells should be used for the isolation of IPN viruses. Amos (1985) and Hill (1976a) state that FHM cells should be used for the isolation of IHN virus and VHS virus.

Some of these cell lines were reconstituted by the supply company and supplied as confluent cultures. This method of obtaining cells was found to be unsatisfactory as some cultures were contaminated on arrival and others had detached from the culture vessels, possibly due to mechanical damage. Another supply company supplied cultures in a frozen state, directly from the ATCC and these cultures were reconstituted in the laboratory. This method of obtaining cells was found to be preferable.

The procedures for the passage and propagation of all four cell lines were successfully established and these cell lines were passaged a number of times (Table 1) with success rates varying from 65% to 89%. Some of the unsuccessful attempts to passage the cells could be attributed to bacterial contamination. The sources of bacterial contamination varied and included contaminated batches of media or ATV and in some cases, contamination during passage or feeding of cells. Bacterial contamination problems in the media were eliminated by storage of the media at room temperature after production for 14 days. Contaminated bottles of medium could be identified by turbidity and were removed. Contaminated ATV was filter sterilized. It was found that stringent sterile procedures had to be observed when passaging or feeding the cell cultures and this greatly reduced contamination problems. The higher success rate obtained with the BF2 cells could be explained by the fact that this was the last cell line to be obtained and some experience in working with cells had been acquired.

All cultures were observed daily and the time required to reach 100% confluency recorded (Table 2 & Fig 5 - 8). No efforts were made to establish optimal incubation temperatures for the

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All cultures were observed daily and the time required to reach 100% confluency recorded (Table 2 & Fig 5 - 8). No efforts were made to establish optimal incubation temperatures for the

different cell lines as this has been done by a number of authors. It has been stated that the optimal incubation temperature for salmonid cell cultures was about 19C (Wolf & Quimby, 1962) and 20C (Wolf, 1979). Pfitzner (1965) stated that gonadal cell cultures of trout will grow at temperatures between 16C to 22C. Wolf & Quimby (1962) found that RTG2 cells at 18C required about 1.3 times longer to reach equal levels of protein as cells held at 24C did.

It was decided to incubate RTG2 cells at between 19C - 22C as most authors state that the optimal temperature for growth of this cell line is in this range. The optimal temperatures for warm water fish has been found to be about 25C (Wolf, 1979), thus the other cell lines were incubated at 25C.

The BF2 cell line was found to be the fastest growing of the four cell lines with an average incubation period of 82 hours. The FHM and BB cells had average incubation periods of 96 hours and 126 hours respectively. The slowest growing of the four lines was the RTG2 line with an average incubation period of 158 hours. The BF2 and FHM cell lines grew fast and seldom had to be fed, thus reducing the chances of bacterial contamination. Most of the cultures of RTG2 and BB cells were fed during incubation. It should be noted that the success rate, when passaging BB cells, was the lowest. As this cell line had to be fed during incubation, the chances of bacterial contamination are high and thus the low success rate. All but the BF2 cells were found to survive for long periods, up to 2 months, if fed with maintenance medium containing 2% FCS. The BF2 cells on the other hand, started degenerating after about 14 days of incubation. The degeneration of this cell line proved problematic when attempting to isolate viruses.

The cells were passaged once they were confluent and in most cases the splitting of the culture to produce three new cultures proved most effective. One 75 cm^2 flask was used to seed three 24 well plates. In the case of the faster growing cell lines (BF2 and FHM), four 24 well plates could be made with equal success.

Samples of all four cell lines were stored in liquid nitrogen for periods varying from two weeks to over 2 years and were successfully reconstituted (Table 3). High success rates were obtained when the BF2, BB and FHM cell lines were frozen and reconstituted (65,91%, 70,00% and 100% respectively). In the case of the BF2 cells, 29 out of 44 attempts to reconstitute these cells were unsuccessful. Four of these could be attributed to bacterial contamination. The remaining unsuccessful attempts were attributed to problems with the FCS. A batch of locally produced FCS was obtained and it was discovered that this serum did not support the growth of the cells. The BF2 cell line was the only cell line which was reconstituted using this batch of serum. Low

success rates were also recorded with the BB cell line (Table 3). They often took very long to attach and reach 100% confluency after freezing. These cultures had to be fed often and bacterial contamination accounted for the loss of three cultures. The RTG2 cells had the lowest success rate (Table 3). This was due mainly to the fact that some of the frozen cultures originated from contaminated cultures. The contamination was suppressed by the antibiotics in the growth medium and only became apparent after the cultures had been frozen and reconstituted. Higher success rates were obtained with other batches of RTG2 cells, although some cultures did not attach to the culture vessel, thus possibly indicating cellular damage during freezing. Other factors, such as toxicity of FCS or reagents used for cell culture were ruled out as medium, FCS and reagents from the same batches supported cell growth in other cultures.

All four fish cell lines were successfully passaged and propagated, although some problems with bacterial contamination were experienced in the early stages. These cell lines could also be stored in liquid nitrogen and successfully reconstituted.

5.2. Virus inoculation, cytopathic effects and virus storage.

The procedures for the inoculation of the fish cell lines with the trout viruses and the study of the cytopathic effects produced by these viruses had to be established before any attempts could be made to isolate viruses from the field.

Samples of IHN virus, VHS virus, herpesvirus salmonis and the VR299, Ab and Sp serotypes of IPN virus were inoculated onto the RTG2, BF2, FHM and BB cell lines. The VR299 and Sp serotypes of IPN virus were found to produce CPE on all four cell lines, while the Ab serotype did not replicate on the FHM cells. This corresponds to the results found by Vestergard-Jorgensen & Kehlet (1971). IHN virus and VHS virus did not produce CPE on the BB cells (Table 4 & Fig 10 - 12) while herpesvirus salmonis only produced CPE on the RTG2 cells (Table 3 & Fig 12). These results correspond to those reviewed by Wolf & Mann (1980) and Amos (1985).

Low success rates were obtained when the cell lines were inoculated with the different serotypes of IPN virus (Table 4). For example, when IPN VR299 virus was inoculated onto RTG2 cells, CPE only occured in 62,5% of inoculations. Similar results were found when this virus was inoculated onto the other cell lines (Table 4). Low success rates were also obtained when the Ab and Sp serotypes of IPN virus were inoculated onto the different cell

lines. There are three possible explanations for these low success rates. The first of these is bacterial contamination which accounted for some, but not all of the unsuccessful attempts. Another possible reason for the low success rates could be a build-up of defective interfering (DI) particles. Nicholson & Dunn (1974) proposed that the inhibition or reduction of IPN VR299 virus replication could be caused by particles which are similar to the IPN VR299 virus. They termed these defective interfering particles and stated that they were similar to the IPN virus because of the interfering particles' susceptibility to immune serum, UV irradiation and the sedimentation properties. DI particles have been reported from many viral systems (Huang, 1973 as cited in Nicholson & Dunn, 1974) and these particles generally possess normal viral structural proteins, but lack some segment of the genome. The different serotypes of IPN virus had been passaged a number of times, thus possibly allowing for a build-up of DI particles (Nicholson & Dunn, 1974) and this could adequately explain the low success rates obtained when inoculating cells with IPN virus. However, it is well known that dilutions of stock samples of viruses where DI particles are present will result in the increased production of infectious standard viruses than if the undiluted stock samples were inoculated onto cells (Huang, 1973, as cited in Nicholson & Dunn, 1974). In most cases cells were inoculated with a 1/2000 dilution of the reference viruses. This high dilution factor should have eliminated most of the problems which could have been caused by DI particles. In some cases, however, particularly when attempting to increase the titre of the viruses, undiluted culture fluid was directly passaged onto new cell cultures, thus increasing the possibilities for interference by DI particles.

The third possible reason for the low success rates could be the fact that IPN virus grown on a particular cell line may not grow as well on another line (Nicholson, Thorne, Janicki & Hanson, 1979). This is particularly true when the virus was grown on RTG2 cells and then passaged onto BF2 or FHM cells (Nicholson *et al*, 1979). The samples of reference viruses were all passaged on RTG2 cells after arrival. It is thus possible that the explanations offered by both Nicholson & Dunn (1974) and Nicholson *et al* (1979) for low success rates when inoculating cell cultures with IPN virus may be valid in this case.

A strange and unexplained phenomenon was observed with the FHM cell line. From the literature it was found that the FHM cell line is the line of choice when attempting to isolate IHN virus and VHS virus (Hill, 1976a). The VR299 and Sp serotypes of IPN virus have been reported to replicate on the FMH cells (Nicholson *et al*, 1979). This cell line was, however, reported as being refractory to some of the serotypes of IPN virus including the Ab serotype (Vestergard-Jorgensen & Kehlet, 1971; Nicholson *et al*, 1979; MacDonald, *et al*, 1983). In the early stages of this project, IHN virus, VHS virus and the VR299 and Sp serotypes of IPN virus were found to replicate on this cell line (Fig 10C,

11C & D & 12C). This corresponds to the work by Hill (1976a), Amos (1985) and Nicholson et al (1979). It was found that the Ab serotype of IPN virus did not grow on this line, which corresponds to the work of Vestergard-Jorgensen & Kehlet (1971). Then, inexplicably, the FHM cell line became refractory to all these viruses, hence the low success rates obtained (Table 4). This 'loss of susceptibility' occurred suddenly and could not be explained by passage number of the cells, pH changes, temperature changes or any changes in materials or procedures. There are reports in the literature that infection of cells by Mycoplasma sp. particularly Mycoplasma laidawii may result in RTG2 and FHM cells becoming resistant to the viruses. It is however unlikely that the sudden loss of susceptibility of the FHM cells could be explained by a mycoplasma infection. Samples of the cells were sent to the Mycoplasma Section of the Veterinary Research Institute, Onderstepoort, but no Mycoplasma sp. could be isolated or detected. None of the other cell lines which were in use at the time developed any loss of susceptibility, even though the same medium was used in all cases. Hill (1976a) states that mycoplasma infection may result in loss of susceptibility in both RTG2 and FHM cells. There was no loss of susceptibility in the RTG2 cells. Frozen cultures of FHM cells which were not refractory to the viruses before freezing were reconstituted, but it was found that the viruses would not produce CPE on these cells either. This phenomenon could not be explained by any of the fish pathology laboratories approached and none of them had experienced anything similar.

As the fastest growing of the viruses under investigation, VHS virus had an average incubation period of 39 hours, any cytological changes observed in infected cultures before about 35 hours post infection (PI) are unlikely to be as a result of virus replication and are more likely to be due to toxicity of the samples. The fluid from any culture showing cytological changes before 35 hours PI should be removed, diluted to eliminate the toxicity and reinoculated onto cell cultures.

If the average incubation periods for the different cell lines, their sustained susceptibility to the trout viruses and the average incubation periods for the development of CPE are considered, the cell lines of choice for the isolation of viruses from trout in South Africa are the BF2 and RTG2 cell lines.

It should be noted that 100% success rates were not always obtained when attempts were made to reconstitute stored virus samples (Table 5 - 8). When viruses were stored in liquid nitrogen (Table 7) only VHS was reconstituted with less than 100% success. Only two cultures were unsuccessful and these were due to bacterial contamination. When the different viruses were stored at -20C (Table 6), none were reconstituted with 100% success. In the case of IHN virus, VHS virus and herpesvirus salmonis, all the unsuccessful attempts were due to bacterial

contamination. As with the inoculation of cells with IPN virus, which was discussed above, there are three possible reasons for the unsuccessful attempts to reconstitute stored IPN viruses (Table 5 & 6). Bacterial contamination is one reason, with the interfering effects of DI particles on the replication of the stored viruses being another possibility. This may account for unsuccessful attempts with viruses stored in 50% glycerol, but it is unlikely that DI particles prevented replication of IPN viruses which had been stored at -20C. Nicholson & Dunn (1974) reported that one cycle of freezing and thawing greatly reduced the interference of DI particles. It is thus likely that the explanation of Nicholson et al (1979), i.e. the inability of IPN virus grown on RTG2 cells to replicate on BF2 and FHM cells, is the more likely explanation for the low success rates when reconstituting IPN viruses stored at -20C. The total number of unsuccessful attempts to reconstitute IPN viruses stored in 50% glycerol is higher than for samples stored at -20C. As Nicholson & Dunn (1974) found that one cycle of freezing and thawing destroyed DI particles, the higher success rate obtained when frozen virus samples were reconstituted was expected as only those virus samples which were grown on RTG2 cells, reconstituted and inoculated onto other cell lines would be unsuccessful. However, the success rate for the reconstitution of IPN viruses stored in liquid nitrogen is 100%. All these attempts were, however, made on RTG2 cells. In this case, all DI particles would have been destroyed by freezing and thawing and the problem of virus grown on RTG2 cells not growing on the other cell lines was eliminated and thus the 100% success rate. Viruses which had been freeze dried were all reconstituted with 100% success (Table 8). All of these reconstitution attempts were made on RTG2 cells, thus eliminating the problem of interference as discussed previously.

5.3. Plaque assay.

Some difficulties were initially experienced with the plaque assay technique, as seen by the low success rates obtained (Table 9). The plaque assay is a useful technique which can be used to determine the titre of a virus sample in plaque forming units (pfu) per ml, thus further attempts to establish the technique were made. This assay is also extensively used in cross neutralization work. After several unsuccessful attempts to perform the plaque assay, success was achieved by careful monitoring of the temperature of the molten agar. It is believed that all the unsuccessful attempts of this assay were due to damage of the cell sheet resulting from high temperatures of the molten agar. The RTG2 cells have an optimal incubation temperature of 19C, with 26C being the maximum temperature tolerated (Wolf & Quimby, 1962). The cells were found to die after 24 hours at 30C (Wolf & Quimby, 1962). The molten agar has to be poured at about 39C to 40C to prevent solidification. If the agar had not cooled down to

as low as possible before being poured, the cells were damaged by the high temperature. The most practical system to do plaque assays was to melt only enough agar to cover two or three cultures at a time. If larger volumes of molten agar were used, the temperature of the agar used to overlay the first two or three cultures was too high. If the large volume of agar was allowed to cool to 39C before overlaying the first cultures, the agar would solidify before all the petri dishes or wells in a 6 well plate could be overlayed. The plaque assay was thus successfully carried out by careful control of the temperature of the molten agar.

There is a commercially available low temperature gelling agarose which would make the plaque assay easier to perform when working with temperature sensitive cells. No efforts were made to obtain and use this agarose as success using normal agarose was obtained by careful monitoring of the temperature. All the results required for this project were successfully obtained using normal agarose. The main objective of this project was the isolation of viruses from fish in South Africa and this could be done without the plaque assay. If extensive cross neutralization work was undertaken, low temperature gelling agarose would be most useful.

5.4. Virus concentration and electron microscopy

Virus particles were seen in ultra-centrifuged, negatively stained culture fluid from cells infected with IPN VR299 virus (Fig 14) and in ultra-thin sections of infected cells (Fig 15). The size of the virus particles was calculated and a diameter of between 52 nm and 64 nm was found. The average particle size was calculated to be 55 nm. This was calculated from 24 micrographs of all three serotypes of IPN virus at different magnifications. It was found that the VR299, Ab and Sp serotypes of IPN virus were all of a similar size and no difference in size between the different serotypes of IPN virus could be seen from the micrographs. The average particle size, as calculated above, corresponds to the diameter of IPN virus found by Moss & Gravell (1969) and Hill (1982). The virus particles in Fig 14 and 15 are clearly icosahedral, which corresponds to the morphology of IPN virus (Hill, 1982). From these results and the results of the serological tests it can be concluded that the virus particles seen in the EM studies are IPN virus.

It should be noted that in the negatively stained preparation there are a number of apparently empty virions. No apparent reason could be found for the high percentage of these apparently empty virions. It is unlikely that these are the DI particles described by Nicholson & Dunn (1974) as it has been proposed that DI particles have normal viral structure, but lack segments of the genome (Huang, 1973; as cited in Nicholson & Dunn, 1974).

5.5. Serological tests.

The procedures for the serological identification of the trout viruses had to be established before any attempts could be made to isolate the viruses from fish in South Africa. Virus specific antibodies against the different viruses were kindly supplied by Dr de Kinkelin (France), Dr Hill (England) and Dr Anderson (USA). Successful attempts were also made to produce antibodies against the VR299 serotype of IPN virus in rabbits.

A variety of serological techniques for the identification of viruses isolated from fish have been established. The techniques most commonly used are the indirect fluorescent antibody test (IFAT) (Piper et al, 1973), serum neutralization test (Lientz & Springer, 1973) and enzyme-linked immunosorbent assay (ELISA) (Nicholson & Henchal, 1978). Other techniques, such as immunoperoxidase assay (Nicholson & Henchal, 1978), complement fixation test (Finlay & Hill, 1975), viral induced bacterial co-agglutination test (Kimura, et al, 1984) and immunoblot assay (McAllister & Schill, 1986) have been developed but so far these techniques are not in general use. The VIBCA and immunoblot assay are recent additions to the serological techniques available to identify fish viruses. The VIBCA test is limited in sensitivity (Kimura et al, 1984) but the speed with which this test can be done will probably ensure wide spread use. The immunoblot assay will undoubtably become a standard technique for fish virus identification due to its sensitivity and specificity.

Although ELISA is widely used in fish virology, this technique was not used in South Africa. The trout industry in this country is very small and only a small number of samples for examination would thus be available. This small number of samples does not warrant the time and expense required to establish an ELISA system. Should a commercial ELISA system for the trout viruses become available in South Africa, it would be obtained and used.

The main objective of this work was to ascertain the virological status of trout in South Africa. At the start of this project, none of the trout viruses had ever been isolated in South Africa and it was believed, from the lack of clinical evidence of viral diseases in trout, that none would be isolated. It was thus decided that IFAT and the serum neutralization test would be adiquate to identify any viruses which may be isolated.

5.5.1. Indirect fluorescent antibody technique

The first of the serological tests to be done was the indirect fluorescent antibody technique (IFAT). It was found that this test was often difficult to interpret due to non-specific reactions, thus making the test very subjective. Negative controls for this technique were extremely important and were used to ascertain the levels of non-specific staining. Extreme care was also needed when performing the test and all stages had to be washed well to reduce the amount of non-specific staining. Excessive washing, on the other hand could also be problematic and could result in the washing off of either the viral specific antibodies or the FITC labelled antibodies. In two attempts to do IFAT on cells infected with IPN VR299 virus, no significant differences could be detected between the test cells and the controls. This was possibly due to incomplete washing which resulted in excessive non-specific staining. These tests were recorded as unsuccessful. Similarly, one test each, when cells were infected with IPN Ab virus and IPN Sp virus, was regarded as unsuccessful, resulting from excessive non-specific staining.

The results were often very difficult to interpret when cells infected with a particular serotype of IPN virus were treated with viral specific antibodies against a different serotype of IPN virus. Fluorescence was obtained in all cases as there is some cross reaction between the different serotypes of IPN virus. A quantitative analysis of the fluorescence patterns was not possible due to this cross reaction. The serological identification of IPN virus to the serotype level was thus very difficult by IFAT. This technique was, however successfully used to differentiate between IPN virus and VHS virus.

The commercial availability of good quality FITC labelled anti-rabbit makes the indirect method the method of choice. The direct method can also be done (Piper *et al*, 1973) in which case fluorescent dyes would have to be conjugated to the viral specific antibodies, which is a laborious task requiring standardization.

5.5.2. Immunoperoxidase assay

Although insufficient repetitions of the immunoperoxidase assay were carried out to make meaningful analyses of the results, the indications were that this technique was very similar to IFAT. The test was only carried out twice on IPN VR299 virus infected cells and twice on VHS virus infected cells, but it was found

that the interpretation of results was difficult due to non-specific reactions. This corresponds to work done by Nicholson & Henchal (1978). Although Nicholson & Henchal (1978) found that both the direct and indirect methods were efficient techniques for the serological identification of IPN virus, these techniques have not been widely used. As no significant differences were found between IFAT and the indirect immunoperoxidase assay, it was decided to use IFAT.

5.5.3. Serum neutralization test

The serum neutralization test is probably the most widely used technique for the serological identification of fish viruses (Hill et al, 1981). This technique was used for the serological identification of samples of the reference viruses (Table 13). Neutralization indices of over 6 were found when samples of the different serotypes of IPN virus and VHS virus were reacted with their homologous antisera. Neutralization indices of over 6 were also found when the different serotypes of IPN virus were reacted with a group polyvalent serum made by mixing equal quantities of anti IPN VR299, anti IPN Sp and anti IPN Ab. As most of the European isolates of IPN virus fall into either the Ab or Sp serotype group (Dorson, 1982) and most of the North American isolates fall within the VR299 group (Dorson, 1982), the group polyvalent antiserum used in South Africa would be capable of neutralizing most of the IPN viruses likely to occur in South Africa. The group polyvalent antiserum used in South Africa was improved by including antiserum against the West Buxton serotype of IPN virus as the West Buxton serotype results in a much more homogeneous clustering of USA isolates than the VR 299 serotype (Hill, 1985 personal communication).

From cross neutralization work (Table 13) it was found that there was some degree of cross neutralization between the different serotypes of IPN virus. The neutralization indices indicate that the Sp serotype of IPN virus is more closely related to the VR299 serotype than to the Ab serotype. Although neutralization was found in all cases when different serotypes of IPN virus were mixed with specific antibodies, the neutralization indices were far lower than with the homologous virus and antibody combination.

5.5.4. Viral induced bacterial co-agglutination test (VIBCA)

The viral induced bacterial co-agglutination test (VIBCA) (Kimura et al, 1984) was evaluated as another method for the serological identification of IPN virus (Table 16). Kimura et al (1984) used this method to serologically identify IPN viruses, IHN virus and Oncorhynchus masou virus (OMV) but no reports on the use of this method for the serological identification of VHS virus could be found. It was found in this laboratory that VIBCA could also be used for the serological identification of VHS virus without any modifications to the techniques used by Kimura et al (1984). In this study the VIBCA test was only used to identify cell free antigens which had been cultured in cell cultures. Kimura et al (1984) also reported great success using this technique to identify viral antigens extracted directly from diseased fish.

The main advantage of this technique is its rapidity. In this laboratory results could be obtained about 15-20 minutes after the first indication of CPE on infected cells. If this technique can be used to detect viral antigens extracted directly from diseased fish as claimed by Kimura et al (1984), a presumptive diagnosis could be made in less than a hour after the fish had been submitted to the laboratory, thus facilitating rapid introduction of methods to reduce the spread of the virus. One possible limitation of this technique is that high virus titres are needed for a successful result. It was found that a virus titre of higher than 3×10^6 TCID50/ml was required. This corresponds favourably to results obtained by Kimura et al (1984). They found that the minimum amount of cell free antigen required for a positive result varied between $10^{5,9}$ and $10^{7,7}$ TCID50/ml. Kimura et al (1984) did, however prove that this technique was useful for the diagnosis of IPN in fish. They were able to detect IPN virus antigen in rainbow trout, coho and amago salmon from sites in Japan using VIBCA. IPN virus was isolated from these fish by conventional methods thus confirming the results obtained with the VIBCA test. This technique was also carried out successfully using S. aureus which had been sensitized with group polyvalent antiserum against IPN viruses (Table 16).

The specificity of this test was verified by the fact that agglutination was only found when the virus samples were mixed with S. aureus Cowan strain A which had been sensitized with homologous antibodies. No agglutination was seen when unsensitized bacteria were used or when the virus and the antibodies used to sensitize the bacteria were not homologous.

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

The Fc binding characteristics of Protein A (Kronvall, Seal & Finstad 1970), which is produced in the cell membrane of S. aureus Cowan strain A (Verwey 1940) has been used to develop a new technique called the immunostaphylococcus-protein-A (ISPA) test. This technique is similar to the fluorescent antibody technique and immunoperoxidase test and, as with these techniques, there is a direct and an indirect method. It is believed that the direct immunostaphylococcus-protein-A test is superior to either IFAT or immunoperoxidase assay in that this test is easily quantified by counting the number of attached bacteria per cell. Inadequate washing during any of these three procedures results in residues of the markers which often make the test difficult to interpret. In the case of direct ISPA, quantification of this residue can be obtained, thus making the test less subjective.

The techniques for stabilization and sensitization of the S. aureus Cowan strain A were the same as those described by Kimura et al (1984). The experiments carried out to test whether the virus specific antibodies had bound to the protein A of formalized S. aureus Cowan strain A were, as far as could be ascertained, original work. The theory behind the sensitization controls was very simple. Samples of S. aureus Cowan strain A were sensitized with rabbit raised viral specific antibodies. When the sensitized bacteria were heat fixed on a glass slide and overlayed with FITC labelled anti-rabbit, these fluorescent labelled antibodies recognized and bound to the rabbit raised antibodies which were bound to the Protein A of the bacteria and fluorescence was seen (Fig 20). It could, however, be argued that as Protein A binds IgG, it could also bind FITC labelled IgG, thus the fluorescence would be non-specific. To clarify this, samples of unsensitized S. aureus Cowan strain A were stained with FITC labelled anti-rabbit IgG. In these cases no rabbit raised antibodies were attached to the Protein A sites and these sites were thus available to other IgG molecules. It was, however found that if unsensitized S. aureus Cowan strain A were overlayed with FITC labelled anti-rabbit, no fluorescence was seen. A possible explanation for this is that Protein A binds to the Fc region of the IgG molecule (Kronvall, et al, 1970). FITC also binds to the Fc fragment of IgG molecules, thus blocking the Fc region and preventing the FITC labelled IgG from binding to the Protein A of the bacteria.

Samples of rabbit raised viral specific antibody sensitized bacteria and unsensitized bacteria were overlayed with FITC labelled anti-mouse. In the case of the sensitized bacteria, the FITC labelled anti-mouse did not recognize the rabbit raised antibodies attached to the bacteria and thus did not bind to them. The FITC labelled anti-mouse was washed off and no

fluorescence was seen. As was the case above, no fluorescence was seen when unsensitized bacteria were overlayed with anti-mouse for the same reasons as discussed above. As fluorescence was only seen when bacteria which had been sensitized if rabbit raised antibodies were overlayed with FITC labelled anti-rabbit, it can thus be concluded that IFAT is a useful technique for determining if the bacteria were successfully sensitized. All of these experiments were repeated using peroxidase labelled anti-rabbit or peroxidase labelled anti-mouse. A brown precipitate was only found when the bacteria which had been sensitized with rabbit raised antibodies were overlayed with the peroxidase labelled anti-rabbit (Fig 21).

It can thus be concluded from the sensitization controls that formalized samples of S. aureus were successfully sensitized with virus specific antibodies. These virus specific sensitized samples of S. aureus could be stored at 4C until needed.

The direct immunostaphylococcus-protein-A test (ISPA) was developed during the course of this study.

The number of attached bacteria per cell was counted and it was found that the average number of attached bacteria per virus infected cell, reacted with S. aureus Cowan strain A sensitized with homologous antibodies was about an order of magnitude greater that when the same sensitized bacteria were reacted with uninfected cells (Table 14).

It was found, from the cross neutralization studies, that the number of attached bacteria per infected cell was still significantly higher than with uninfected control cells (Table 14). As with the serum neutralization test, the Sp serotype of IPN virus was antigenically more closely related to the VR299 serotype than to the Ab serotype. This technique was also suitable for the serological identification of IPN virus when the bacteria were sensitized with group polyvalent antiserum against IPN virus.

The specifity of this technique was tested by reacting IPN virus infected cells with anti VHS sensitized bacteria, or vice versa. The number of attached bacteria per infected cell was similar to the number attached to the uninfected cells.

It is felt that this technique is superior to IFAT and the immunoperoxidase assay because, although attached bacteria were seen in the negative controls, they were easily quantified, thus making this technique less subjective.

There is one precaution which must be observed when performing the direct ISPA test. Under no circumstances should the cell sheet be allowed to dry once the sensitized bacteria have been added. If the cell sheet dries out, the bacteria will attach irrespective of the presence of viral antigen. Once the technique has been completed, however the cell sheets can be allowed to dry out for storage and reference.

5.5.6. Indirect ISPA

The indirect ISPA method was also established during the course of this study. In the indirect method, samples of S. aureus Cowan strain A were sensitized with samples of anti-rabbit IgG. The results obtained when the indirect ISPA test was performed were similar to those obtained with the direct method (Table 15, Fig 24 & 25).

It must be noted that on average, the number of attached bacteria per cell is higher in the indirect method compared to the direct method. This is particularly noticeable in the uninfected control cells and cells infected with a virus not homologous to the antibodies used to sensitize the S. aureus. This may be due to incomplete washing of the cells after the virus specific antibodies have been added to the cells. If all the antibodies are not washed off, some of the anti-rabbit sensitized S. aureus will react with this residue and the average number of attached bacteria will increase. Inadequate washing can also be problematic in the IFAT and indirect immunoperoxidase assay, but in the case of the indirect ISPA, this can be quantified, thus making the test less subjective.

5.6. Comparison of serological techniques.

The VIBCA test was the least sensitive of all of the serological techniques which were investigated. The VIBCA test could only be used to detect viruses if the titre of the virus suspension was greater than $3,125 \times 10^6$ TCID 50/ml. There appears to be little difference between the sensitivities of the other tests and all of these could be used to detect a virus with a titre of between $3,125 \times 10^1$ and $3,125 \times 10^2$ TCID 50/ml. As the sensitivities of most of the serological tests are similar, the selection of techniques to use is based on specificity. It was thus decided to use the serum neutralization test and direct ISPA test, as both these tests are very specific. IFAT was used in conjunction with the serum neutralization test before the development of the ISPA test, and this test was used throughout for continuity.

5.7. Isolation and identification of viruses from fish in South Africa.

The prime objective of this study was to determine if IPN virus, IHN virus, VHS virus, herpesvirus salmonis are present in South Africa. One approach was to attempt to isolate and serologically identify the viruses from various populations of fish, both diseased and asymptomatic. Another approach was to collect samples of blood from fish for virus neutralization test on the serum thus determining the presence of neutralizing antibodies, which would indicate past exposure to the viruses.

Viruses were isolated from 11 of the 135 cases submitted for examination (Appendix 2 & Table 17). All the isolated viruses were serologically identified as IPN virus by IFAT (Table 18). It was found from the serum neutralization test and the direct ISPA test (Table 18) that these viruses were all serologically related to the VR299 serotype of IPN virus. This is particularly noticeable in the direct ISPA test where the number of attached bacteria per cell infected with the isolated viruses and overlayed with anti IPN VR299 sensitized S. aureus Cowan strain A was between 4,7 and 7,5. The number of attached bacteria per infected cell was only between 2,7 and 2,8 when anti IPN Ab sensitized S. aureus was used and between 3,8 and 4.0 when anti IPN Sp sensitized S. aureus was used. The number of attached bacteria per cell was only between 0,2 and 0,4 when cells infected with the isolated viruses were overlayed with anti VHS sensitized S. aureus. More bacteria per cell attached when the S. aureus were sensitized with anti IPN VR299 than any other, thus indicating that the isolated viruses were related to the VR299 serotype of IPN virus. Samples of these viruses were sent to Dr Hill of the Fish Disease Laboratory, England who confirmed that the isolated viruses were indeed the VR299 serotype of IPN virus.

In case No F33/85, a low incidence of mortality was found in trout which had been imported from Denmark by the farm 'Waterval'. The isolation of IPN VR299 virus from these trout imported from Denmark caused much concern as this isolate is a North American serotype and has not been reported from Europe or Scandinavia (Hill, 1988 personal communication; de Kinkelin, 1988 personal communication). Upon further investigation it was found that fish which had been imported from the USA had been kept in the ponds just prior to the import of the Danish fish. A further 50 samples of both the Danish and American fish were collected and virus isolation attempted. Viruses were isolated from four pools of five fish each, collected from the American fish, while six pools collected from the Danish fish showed CPE. It would thus appear that the virus was introduced onto the site via infected ova imported from the USA.

Case number F100/86 (Table 17), involving fingerling rainbow trout was of interest because it was the only time that IPN virus was isolated from a site which reported high mortalities. This caused some concern because none of the other sites from which IPN virus was isolated reported high mortalities and there are no reports in the literature of mortalities in fingerling rainbow trout as a result of infection by IPN virus. Elazhary et al (1976) did, however, report on stress mediated mortalities in yearling rainbow trout as a result of IPN. Upon further investigation, however, the fish pathogenic bacterium, Aeromonas salmonicida, was isolated from moribund fish (Bragg, unpublished data, 1986). It is proposed that IPN virus was not responsible for the high mortality rate in these fingerlings and that the fish were carriers of the virus. The mortalities were as a result of furunculosis, caused by A. salmonicida. The occurrence of IPN virus in fingerlings which have survived an IPN virus infection and have become carriers of the virus is well documented (Mangunwiryo & Agius, 1988; Yamamoto, 1975a; 1975b).

The remaining cases listed in Table 17 were all part of a survey of trout produced on sites which sell fingerlings for restocking. The survey was undertaken as a result of the isolation of IPN VR299 virus from cases F33/85 and F100/86.

Large numbers of viscera samples from asymptomatic fish were collected on a number of trout farms (Appendix 3). A virus was isolated from samples of adult fish collected on the farm 'Fisantekraal' in the Cape Province during June 1986. A virus was also isolated from adult fish collected from the farm 'La Rochelle' in the Sabie area of the Transvaal. Another virus was isolated from fry collected from a TPA hatchery in Lydenburg. These samples were collected during July 1986 (Table 19).

All three isolates were serologically identified as being similar to the VR299 serotype of IPN virus (Table 20). The CPE caused by these isolates were indistinguishable from those caused by the virus isolated from case F33/85 (Fig 26). Two of these sites, i.e. Fisantekraal and La Rochelle had a history of imports from a hatchery in the USA, while the other site did not import from the USA. This site is, however, in close proximity to a number of infected sites and the virus could have been introduced to this site via a vector such as fish eating birds. Sonstegard & McDermott (1972) proposed that IPN virus could be transmitted by fish eating birds, such as the great horned owl (Babo virginianus). Fish eating birds in South Africa, such as the hamerkop (Scopus umbretta) or the white breasted cormorant (Phalacrocorax carbo) could have carried IPN virus to this site. Although virus was isolated from fry, which have been reported to be susceptible (Hill, 1982), no mortalities were reported. Similar situations have been reported before in other countries. IPN virus was isolated from trout from a hatchery in England which reported no mortalities (Hill, 1982). Upon laboratory

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investigation, this isolate was found to be highly pathogenic to rainbow trout fry. It would thus appear that other factors such as environment or physiological state of the fish play a role in the outcome of infection with IPN virus.

Samples of ovarian fluid were also collected on trout farms during the normal stripping operations. A total of 2 892 ovarian fluid samples was collected from 13 farms throughout South Africa (Appendix 4). It was found that samples of ovarian fluid collected from the farm 'Aquacultura' were infected with a virus. This site also had a history of ova imports from the USA. The virus was identified as IPN virus by IFAT and found to be serologically similar to the VR299 serotype by the serum neutralization and the direct ISPA test (Table 21).

A total of 5 200 eyed trout ova from 22 different sources, most of which had been imported into South Africa were examined (Appendix 5). A virus was isolated from one batch of about 200 eyed trout ova imported from the USA in November 1985. These ova were imported from the same site in the USA which sent ova to all the other sites in South Africa, except the TPA hatchery at Lydenburg, from which IPN VR299 virus was isolated. Samples of the ova were collected at the airport and shipped directly to the Veterinary Research Institute. These ova did not come into contact with any local fish, or fish products. It was thus concluded that the virus was isolated from the ova imported from the USA. The virus was serologically identified as the VR299 serotype of IPN virus by the serum neutralization test and the direct ISPA test (Table 22) and these results were confirmed by Dr Hill.

The fact that all of the viruses isolated from trout in South Africa were serologically identified as the VR299 serotype of IPN virus, plus the fact that this virus was also isolated from a consignment of ova imported from the USA, indicate that the virus was introduced into this country via ova imported from the USA. All the sites in South Africa, except one, had a history of recent ova imports from the same site in the USA, thus enhancing the argument that this virus was imported from the USA. The possibility that this virus was introduced from Europe is most unlikely as the VR299 serotype of IPN virus has never been isolated in Europe (Hill, personal communication, 1988; de Kinkelin, personal communication, 1988).

5.8. Detection of antibodies in fish serum

Antibodies capable of neutralizing the VR299 serotype of IPN virus were detected in fish serum collected in South Africa (Table 23). IPN VR299 virus had been isolated from fish collected on all sites from which neutralizing antibodies were found.

The fact that antibodies were only detected in sites in South Africa from which viruses were isolated must be seen as conclusive evidence of the exposure of these fish to the virus. This completely rules out any possibility of the isolated viruses originating from laboratory contamination of samples with IPN VR299 virus.

Author Bragg Robert Richard
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