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Alternative cell line for the isolation of salmonid alphavirus-1

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ABSTRACT

Salmonid alphavirus (SAV) has recently become an economically important pathogen in salmonid aquaculture in Europe. Subtype SAV-1 causes salmon pancreas disease (SPD) in Atlantic salmon in Scotland and Ireland, and was first isolated on Chinook salmon embryo-214 (CHSE-214) cells in 1995 in Ireland; several established cell lines have since been tested for viral growth, although the ability of these cell lines to support primary virus isolation has not being examined. In the present study, CHSE-214, Chum salmon heart -1 (CHH-1) and Salmon head kidney -1 (SHK-1) cell lines were evaluated for isolation of SAV-1 from kidney samples of experimentally infected Atlantic salmon (*Salmo salar*). The presence of infection in these samples was confirmed both by cell culture and reverse transcription polymerase chain reaction (RT-PCR). Homogenates of kidney from fish 3 days post-infection (p.i.) were inoculated onto the three cell lines and the development of a cytopathic effect (CPE) recorded. The CHH-1 cells produced a rapid CPE from Day 6 p.i., while the CHSE-214 cells showed the presence of a CPE from Day 10 p.i. In comparison, a CPE developed much later in the SHK-1 cells, from Day 20 p.i. The virus was successfully isolated on all three cell lines in subsequent passages, indicating that CHSE-214, CHH-1, and SHK-1 cells can be used for the isolation and culture of SAV-1. The CHH-1 cell line, however, has proven the most useful, since the CPE developed the quickest in this cell line.

Keywords: Cell culture, CHSE-214, CHH-1, PD, SAV-1, SHK-1

YFIRLIT

Val á frumulínum til einangrunar á Salmon Alphavirus-1 laxaveirunni

Á síðustu árum hafa efnahagsleg áhrif sýkinga með laxfiskaveirunnar Salmonid alphavirus -1 (SAV-1) verið að aukast í laxfiskaeldi í Evrópu. Veiruna tókst fyrst að einangra 1995 og var það gert í Chinook salmon embryo-214 (CHSE-214) frumum. Síðan þá hafa nokkrar frumulínur verið reyndar til að rækta veiruna án þess að næmni þessara frumulína hafi verið skoðað m.t.t. veirueinangrunar úr sýktum fiski. Í þessari rannsókn

var ræktunarnæmni frumulínanna CHSE-214, Chum salmon heart -1 (CHH-1) and Salmon head kidney -1 (SHK-1) skoðuð með sýkifloti úr tilraunasmituðum atlandshafslaxi (*Salmo salar*). Veirueinangrun og erfðamögnun (RT-PCR) var notuð til að staðfesta að sýni væru veirusýkt. Sýkiflot frá nýrnavefjasúpu úr fiskum þrem dögum eftir sýkingu var notað til að sýkja frumugerðirnar þrjár og var fylgst með hvenær veiruskemmdir kæmu fram í frumunum. Veiruskemmdir komu fyrst fram í CHH-1 frumunum eða frá 6. degi eftir sýkingu. Í CHSE-214 sáust veiruskemmdir fyrst frá 10. degi og í SHK-1 frumunum frá 20. degi. Veirueinangrun tókst í öllum frumulínunum þrem eftir umrækt, sem bendir til þess að CHSE-214, CHH-1 og SHK-1 frumur eru vel nothæfar til einangrunar á SVA-1 veirunni. Hinsvegar reyndist CHH-1 frumulínan notadrýgst þar sem ummerki veirusýkingar komu fyrst fram í þessari frumulínu.

INTRODUCTION

Salmonid alphaviruses (SAVs) are a group of recently characterized fish viruses which cause disease in farmed salmonids in Europe (McLoughlin & Graham 2007) and altogether 6 different subtypes have now been identified (Hodneland & Endresen 2006, Fringuelli et al. 2008). Pancreas disease (PD) was the first condition associated with this group of viruses, reported in Scotland in 1976 (Munro et al. 1984), although the infectious nature and viral etiology involved was not confirmed until nearly two decades later by isolating the virus in cell culture by co-culturing infected kidney with Chinook salmon embryo cells (CHSE-214) (Nelson et al. 1995). The etiological agent has subsequently been named Salmonid Alphavirus-1 (SAV-1) (Weston et al. 2002). The disease is characterized by severe pathology in the pancreas, heart and skeletal muscles, and can result in mortalities (5-10 % of affected fish) or growth retardation in marketable-sized farmed Atlantic salmon (*Salmo salar* Linnaeus) in Scotland and Ireland. Thus, the disease is now recognized as an economically important disease by the salmon aquaculture industry (Rodger & Mitchell 2007).

Pathology, virus isolation, reverse transcription polymerase chain reaction (RT-PCR), an improved virus neutralization test and immunofluorescent antibody techniques are currently used to identify SAV-1 (Graham et al. 2008). Virus isolation from clinical samples, based on the observation of a cytopathic effect (CPE), is still considered the gold standard in the diagnosis of many aquatic viral diseases and to certify stocks as being disease-free (Anon. 2003). For SAV diagnosis, cultivation and passages of cell culture can be extremely time consuming and require experienced personnel to interpret the changes in the CPE, which is hard to see during the initial stages of development (Nelson et al. 1995). Although different cell lines have been tested for virus growth, little information is available on the use of these cell lines for primary virus isolation from clinically infected material. There is a need for optimized cell culture assays for rapid isolation of SAV-1 for fast and effective virology diagnostics (McLoughlin & Graham 2007). Therefore in the present study three established salmonid cell lines, CHSE-214, Chum salmon heart -1 (CHH-1) and salmon head kidney -1 (SHK-1) cells, were compared for their ability to isolate SAV-1 from experimentally infected Atlantic salmon based on their growth characteristics and CPE development.

MATERIALS AND METHODS

Cell cultures

CHSE-214, CHH-1(Fryer & Lannan 1994) and SHK-1 (Dannevig et al. 1997) cell lines were used for the study. All the chemicals and media were obtained from Invitrogen (Paisley, UK) unless otherwise stated. The former two cell lines were cultured in a growth medium (GM) containing Eagle's Minimal Essential Medium (EMEM), 2 mM L-glutamine, 1% non essential amino acids (NEAA) and 10% (v/v) fetal calf serum (FCS) (Biosera, UK). For virus isolation, maintenance medium (MM) was prepared by adding antibiotics (penicillin (100 IU/ml), streptomycin (100 mg/ml) and kanamycin (100 mg/ml)) to the culture medium, while maintaining the serum concentration at 5% (v/v). Growth media and MM for SHK-1 cells, on the other hand, consisted of Leibovitz L-15 medium with GlutaMax, supplemented with 2

μm L-glutamine, 40 μm mercaptoethanol, 5% (v/v) Australian foetal calf serum, penicillin and streptomycin.

Propagation and maintenance of these cells were carried out following standard procedures. Briefly, 7-8 day old CHSE-214 and CHH-1 cells were trypsinised from the culture flasks, split in a 1:3 ratio and incubated in a 4% CO_2 incubator at 22°C for 18-24 h before infecting with the virus. The SHK-1 cells were split in a 1:2 ratio from 10 day old stock flasks and grown in L-15 medium in 22°C for 48 h before use for virus isolation work.

Virus preparation

Preformed monolayers of CHSE-214 cells (3 x10^7 cells/flasks) prepared in 75 ml flasks (Fisher Scientific, UK) were absorbed with a 1:10 dilution of SAV-1 Irish isolate F93-125 (Weston et al. 2002) kindly provided by Dr. David Smail (Fisheries Research Services, Aberdeen abe) for 4 h at 15°C before adding GM and monitoring daily for the development of a CPE. Flasks were freeze-thawed once at 70°C on Day 9 post-inoculation, and the supernatant was clarified at 3500 g for 10 min at 4°C in an Eppendorf 5804R centrifuge. The clarified supernatant was aliquoted and stored at -20°C. One aliquot was back-titrated onto CHSE-214 cells to determine the 50% Tissue Culture Infective Dose ($TCID_{50}$).

Experimental infection

Disease-free Atlantic salmon (n=50), weighing 32-56 g, were injected intraperitoneally with 0.2 ml of the CHSE-214 cultured SAV-1 preparation, with a dose of 1x$10^{7.33}$ $TCID_{50}$/ml, while the control fish (n=50) were injected with 0.2 ml of clarified CHSE-214 culture supernatant. The fish were maintained in a flow-through system, placing 25 fish into a 50 l circular tank, and monitored twice daily throughout the experimental period of 90 days post-infection (p.i.). Head kidney was aseptically sampled from 5 SAV-1 injected and 5 control salmon at Days 1, 3, 5, 7, 10, 14, 21, 42 and 90 p.i. The tissue was divided into two samples, one for virus isolation by cell culture and one for RNA extraction in order to identify the presence of the virus by RT-PCR. Samples for virus isolation were collected in sterile Bijoux bottles and placed on ice until processed. The remaining material for RT-PCR was fixed in RNA later (Applied Biosystem, Warrington, UK), kept overnight at 4°C and then stored at -70°C after decanting the RNA later.

Preliminary isolation of SAV-1 on CHSE-214 cells

For virus isolation, kidneys were macerated with sterile sand to prepare a 1:50 (w/v) dilution of the kidney homogenate in Hank's Buffered Salt Solution (HBSS) supplemented with 2% FCS, penicillin, streptomycin and kanamycin. The kidney homogenate was clarified by centrifuging at 2000 g for 15 min at 4°C (Eppendorf 5804R). Supernatants were then filtered through a 45 μm filter into sterile universals. The clarified kidney homogenates (100 μl), and 1:100 and 1:1000 (v/v) dilutions of this (100 μl), were absorbed onto preformed (18-24 h old) CHSE-214 cell (2x10^5/well) monolayers in 24 well plates (Fisher Scientific, UK) and incubated at 15°C in a CO_2 incubator. After 4 h MM was carefully added to the monolayers. Plates were observed every other day for 28 days for the development of a CPE.

RT-PCR

Total RNA was isolated from kidney tissues using Tri Reagent (Applied Biosystems, Warrington, UK). The RNA concentration and purity were checked using a NanoDrop 1000 spectrophotometer by estimating the absorbance ratio at 280/260 nm, which was in the range of 1.8-2.0. The quality of the RNA was confirmed by agarose gel electrophoresis.

Total RNA was reverse transcribed using a Reverse-iT™ MAX 1st strand synthesis kit (Abgene, Epsom, UK) according to the manufacturer's instructions. Briefly, 1 μg of total RNA was mixed in a 1:3 ratio with anchored oligo-dT (500 ng/μl) and random hexamers (400 ng/μl), heated for 5 min at 70°C, then kept on ice for 2 min before adding 2 μl 5mM

dNTP, 4 µl 5x first strand synthesis buffer, 1 µl (50 U/µl) *Reverse*-iT™ MAX RTase blend, 1 µl QRTase enhancer and then nuclease-free water to make the final reaction volume up to 20 µl. It was reverse transcribed at 42°C for 60 min with a further 10 min at 75°C for enzyme inactivation and stored at -20°C.

The PCR was performed using a primer pair that produces a 539 base pair (bp) product (Hodneland & Endresen 2006) with modifications to the thermal cycling conditions. For the PCR, 25 µl of reaction mixture containing 2 µl cDNA, 1.25 µl forward (5´-CGGGTGAAACATCTCTGCG-3´) and reverse (5´CTTGCCCTGGGTGATACTGG-3) primers (10 µM/ml), 8 µl nuclease free H_2O and 12.5 µl 2X ReddyMix™ PCR Master mix (composed of 0.625 U Thermoprime *Taq* DNA polymerase, 75 mM Tris-HCL, 20 mM $(NH_4)2SO_4$, 1.5 mM $MgCl_2$, 0.01% (v/v) Tween® 20, 0.2 mM each of dnTP, dCTP, dGTP, and dTTP) (Thermo-Scientific, UK). The thermal cycle conditions consisted of 95°C initial denaturing for 5 min, followed by 40 cycles of 20 s denaturing at 95°C, 30 s annealing at 55°C and 1 min extension at 72°C prior to final extension at 72°C for 5 min. PCR products were visualized on a 1% agarose gel stained with ethidium bromide and viewed under a UV camera.

Comparison of CHH-1, CHSE-214 and SHK-1 cells for virus isolation

Frozen (-20°C) kidney homogenates prepared from samples taken from fish on Day 3 p.i., and which were all CPE positive on CHSE-214 cells in the initial virus isolation, were thawed and absorbed onto the preformed monolayers of CHSE-214, CHH-1 and SHK-1 cells cultured in 24 well plates. Following absorption, MM was added to the CHSE-214 and CHH-1 cells, while L-15 with supplements was added to the SHK-1 cells. Cells were incubated at 15°C observed daily for the development of a CPE over the course of 25 days.

Estimation of virus titre on cell cultures

Cell culture supernatants of the first passage from the infected CHSE-214 and CHH-1 monolayers were harvested after 10 days of incubation and stored at -20°C until the $TCID_{50}$ was estimated for each sample. A tenfold dilution series of each supernatant was made in 96 well plates using HBSS, and these were then absorbed for 1 h onto preformed CHSE-214 and CHH-1 monolayers prepared in 96 well plates (Fisher Scientific), using three replicates for each sample. The $TCID_{50}$ was estimated according to the method described by Spearman and Kaber (Mahy & Kangro 1996), reading the plate after Day 15 of incubation. The titres were compared using a 2-sample t-test.

RESULTS

Virus isolation from cell cultures

Initially CHSE-214 cells were used to isolate the virus from the infected kidney samples to establish the best sampling point (i.e. Days p.i.) to use for the study (Figure 1). All CHSE-214 cells absorbed with kidney homogenate sampled from fish at Day 1 p.i. developed a CPE in the cells from Day 21 p.i. In contrast,

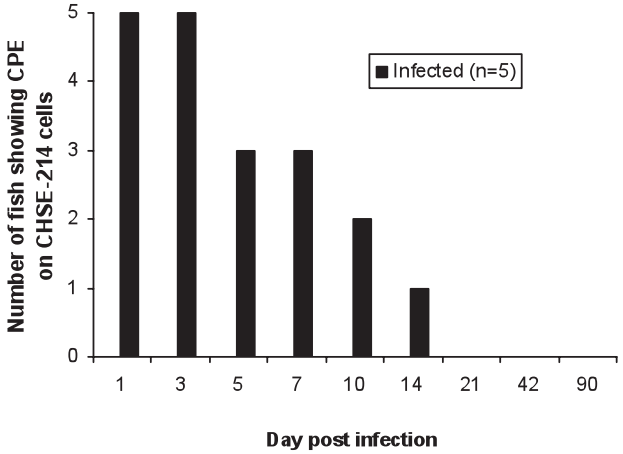

Figure 1. The development of a cytopathic effect (CPE) on CHSE-214 cells with infected kidney sampled at different times (Day 1-Day 90) post infection.

kidney sampled at Day 3 p.i. produced a CPE on CHSE-214 cells from Day 10 p.i. into the cells. Four out of the five infected kidney samples taken at Days 5 and 7 p.i. developed a CPE on cells. However, no CPE was obtained with samples taken from fish at Day 21 p.i. or thereafter, or with any of the control fish.

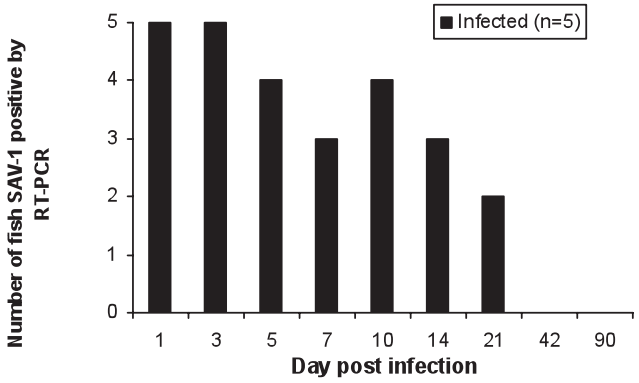

Figure 2. Number of fish tested positive by reverse transcription polymerase chain reaction with kidneys sampled at different times (Day 1-Day 90) post infection.

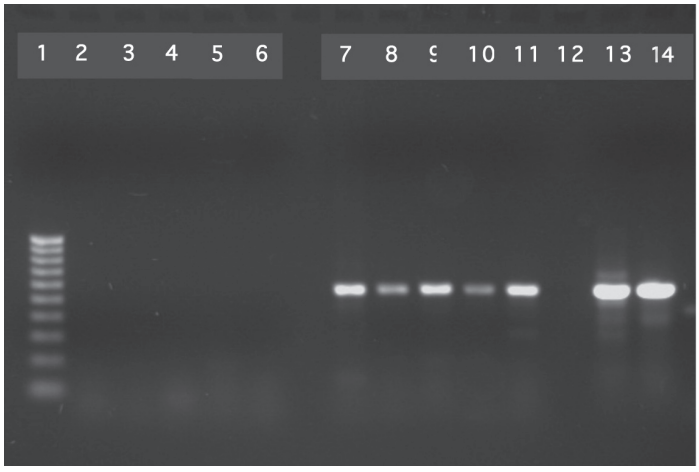

Figure 3. Results of the reverse transcription polymerase chain reaction (on a 1% agarose gel) using RNA extracted from head kidney of fish sampled on Day 3 p.i. Kidney samples from all infected fish gave bands on the gel. Note none were obtained with control fish. Lanes: (1) 100 bp ladder, (2- 6) uninfected fish, (7-11) infected samples, (12) non-template negative control and (13- 14) positive control [CHSE-214 and CHH-1 cell cultures infected with F93-125 Irish isolate]

Detection of SAV-1 in kidney tissues by RT-PCR

The results of the RT-PCR with infected kidney samples are shown in Figure 2. All fish injected with SAV-1 were RT- PCR positive at Days 1 and 3 p.i., while four of the five fish sampled on Days 5 and 10 p.i. were positive. Three of the five fish were PCR positive on Day 7 and Day 14 p.i, while one fish gave a faint band on Day 21 p.i., and thereafter none of the fish were positive. All control fish were negative for SAV-1 by RT-PCR. The RT-PCR results of the kidney samples are shown in Figure 3, in which all infected fish, positive for virus, gave a band at 576 bp on a 1% agarose gel.

Comparison of cell cultures for virus isolation, morphology and titration

The samples taken at Day 3 p. i. were used to compare the suitability of the three cell lines (i.e. CHSE-214, CHH-1 and SHK-1 cells) and hence they resulted in the most rapid CPE in CHSE-214 cells above, and the presence of SAV-1 in the samples was confirmed by RT-PCR (Figure 3). Of the three cell lines absorbed with the infected kidney homogenate, CHH-1 cells gave the earliest CPE, which started to appear in 3 of the replicate samples from Day 6 post-inoculation, and all replicates become CPE-positive by Day 15 p.i. (Table 1). The CHSE-214 cells, the cell line conventionally used for SAV-1 isolation, showed a CPE with 2 of the replicate samples at Day 10 post-inoculation, and all the replicate wells were positive

Table 1. Development of a CPE in Chinook salmon embryo cells (CHSE-214), Chum salmon heart -1 (CHH-1) and Salmon head kidney -1 (SHK-1) cells during primary virus isolation and subsequent two passages of the virus. CHSE-214 and CHH-1 cell cultures were harvested at Day10 post-inoculation on passage 1 and 2, and therefore no data available after this time point. Samples derived from SHK-1 cells were not used for viral titre estimation and experiment stopped after passage 1. P0- Primary inoculation, P1-Passage 1, P2-Passage 2. Each sample representative of individual fish.

	CHSE-214			CHH-I			SHK-1		
Days post inoculation	**P0**	**P1**	**P2**	**P0**	**P1**	**P2**	**P0**	**P1**	**P2**
1	0	0	0	0	0	0	0	0	-
3	0	1	3	0	3	5	0	0	-
6	0	4	5	3	5	5	0	0	-
10	2	5	5	4	5	5	0	1	-
15	4	-	-	5	-	-	0	3	-
20	5	-	-	5	-	-	3	5	-
25	5	-	-	5	-	-	4	5	-

by Day 20. For both these cell lines, a CPE developed faster in subsequent passages of the virus (Table 1). In contrast, the CPE which developed in SHK-1 cells started to appear at Day 20 and only 4 fish were positive for virus in this cell line by Day 25 p.i. However, all samples were CPE-positive in a subsequent passage onto SHK-1 cells (Table 1).

The mean $TCID_{50}$ obtained on CHSE-214 ($1x10^{3.64\pm1.76}$) and CHH-1 ($1x10^{3.36\pm1.4}$) cells were not significantly different ($p < 0.05$) from each other on the first passage of the virus. The virus titre on SHK-1 cells was not measured due to the slower development of the CPE in this cell line.

The morphological appearance of the CPE was similar in both CHSE-214 and CHH-1 cells, which starting as a localized rounding of cells on the surface of the cell monolayer, which then spread over the cell line with cells sloughing off the edges of the affected areas. In contrast SHK-1 cells started to loosen from the monolayer when it started to produce a CPE from Day 20 p.i. (Figure 4).

DISCUSSION

CHSE-214 cells, the cell line commonly used for SAV-1 research and diagnostics (McLoughlin & Graham 2007) has been reported to have several drawbacks with its use for primary virus isolation, such as a delayed CPE development on primary inoculation and the requirement for several passages to develop a visible CPE, thus in some instances leading to an increase in false negative results (Nelson et al. 1995, Jewhurst et al. 2004). The suitability of different cell lines for effective diagnosis of SAV-1 was therefore examined using three established salmonid cell lines (CHSE-214; CHH-1 and SHK-1 cells) to determine if there was a better alternative to CHSE-214 cells for this purpose.

Samples for the experiment were derived from an aquarium-based experimental infection using an isolate obtained from a disease

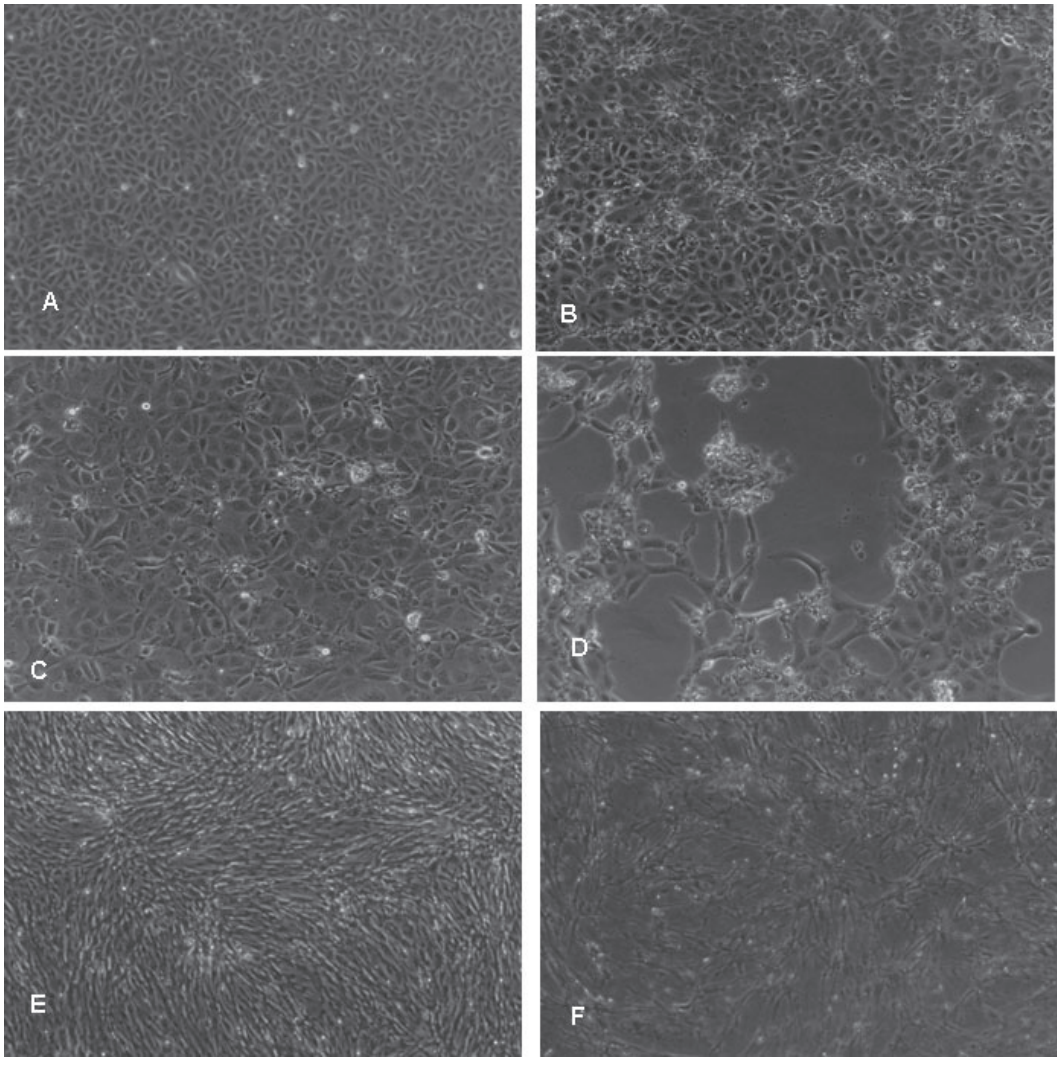

Figure 4. Cytopathic effect (CPE) in three different cell lines inoculated with kidney homogenate sampled Day 3 p.i. from SAV-1 infected salmon. (A) Non-infected Chinook salmon embryo-214 (CHSE-214) cells. (B) infected CHSE-214 cells on Day 6 post-inoculation (p.i). (C) non-infected Chum salmon heart -1 (CHH-1) cells. (D) infected CHH-1 cells on Day 6 p.i. (E) non-infected Salmon head kiney-1 (SHK-1) cells. (F) infected SHK-1 cells on Day 20 p.i.

outbreak in Ireland. The existence of infection in the sampled kidneys over the course of the challenge was confirmed by RT-PCR to ensure the fish had been successfully infected with the virus. Samples from the same kidney tissues used for the RT-PCR were also used for virus isolation on CHSE-214 cells. The kidneys of all fish sampled on Days 1 and 3 p.i. were found to be positive for virus by RT-PCR and cell culture. The kidney samples taken on Day 1 p.i. developed a CPE after Day 20 p.i. In comparison, CPE developed much faster in kidney samples taken at Day 3 p.i., starting from Day 10 during primary inoculation. The CPE which resulted from samples taken at Day 1 p.i. could possibly be due to low levels of infectious virus present in the kidney from the injection itself rather than from virus which

had started to replicate in the fish. Kidney homogenates prepared from Day 3 p.i. samples were used for the subsequent comparative studies on the assumption that these samples had a higher virus load compared to the other sampling points.

With regard to virus isolation, the appearance of a CPE was faster with CHH-1 cells compared with the other two cell lines, although the virus titre was not significantly different to that obtained in the CHSE-214 cells in Passage 1. The SHK-1 cells produced a CPE much later than the other two cell lines, with CPE starting to appear at Day 20 p.i. Graham et al. (2008) also noted a similar result with SHK-1 cells, and found that not all SAV isolates grew in this cell line and always gave a lower titre than CHSE-214.

CHH-1 cells are fibroblast cells that originated from Chum salmon (*Oncorhynchus keta* Walbaum) heart (Lannan et al.1984, Fryer & Lannan 1994) and were found to perform better for primary SAV-1 isolation and propagation of SAV-1 over the conventional CHSE-214 cells, which are epithelial cells that originated from Chinook salmon (*Oncorhynchus tshawytscha* Walbaum) embryo. Furthermore, as the CHH-1 cell line is of cardiac origin, it may be useful for studying the host response to the virus in vitro, since the heart is one of the major target organs of this virus.

SHK-1 cells are macrophage-like, derived from Atlantic salmon head kidney leukocytes (Dannevig et al. 1997), and have been used in immunological, antiviral and host pathogen interaction studies *in vitro* for different viral diseases affecting salmonids (Jensen & Robertson 2002, Martin et al. 2007). The ability of SHK-1 cells to support SAV-1 isolation and propagation would make it a useful tool to study the immunological and antiviral mechanism of the host against this group of viruses, which have not as yet been properly described. CHSE-214 cells, on the other hand, do not have an inherent ability to produce an antiviral effect against the virus (Jensen et al. 2002), which further limits their use for host-pathogen interaction studies *in vitro*.

In a diagnostic laboratory, the faster the cell line develops a CPE, the quicker a diagnosis can be made and the more rapidly a clinical or regulatory response can take place. Therefore this new candidate cell line, CHH-1, has become the cell line of choice for SAV research and diagnostics.

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