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Research Article

Effects of Rainbow Trout (*Oncorhynchus mykiss*) Serum (RTS) on Replication of Infectious Pancreatic Necrosis Virus (IPNV)

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Abstract: A series of experiments were conducted to determine the effects of rainbow trout serum (RTS), taken from rainbow trout (*Oncorhynchus mykiss*) that have never experienced infectious pancreatic necrosis disease, on infectious pancreatic necrosis virus (IPNV) in vitro and in vivo. In vitro, 109 IPNV isolates belonging to various subtypes and having various degrees of virulence were passed in the presence and absence of 1% RTS on rainbow trout gonad (RTG-2) cell line. Moreover, brook trout (*Salvelinus fontinalis*) were challenged with four virulent Buhl subtype isolates passed 11 times in the presence and absence of 1% RTS. Avirulent isolates of IPNV were inhibited from replication on RTG-2 cells even after a single passage in the presence of 1% RTS, whereas isolates having a high degree of virulence were consistently resistant to RTS. Moreover, in vivo the level of inhibition was related with virulence of the virus ($P < 0.0001$). Serum inhibition of RTS on IPNV growth in vivo and in vitro is not dependent on the virus serotype. The effect of RTS presence on virus virulence in virus propagation was variable even in the same serotype.

Key Words: IPNV, 6S, replication, rainbow trout serum, RTS

Gökkuşağı Alabalığı (*Oncorhynchus mykiss*) Serumunun (GAS) Infeksiyöz Pankreas Nekrozu Virüsü (IPNV) Replikasyonu Üzerine Etkisi

Özet: Hiç infeksiyöz pankreas nekrozu geçirmemiş gökkuşağı alabalığından (*Oncorhynchus mykiss*) alınan serumun (GAS), infeksiyöz pankreas nekrozu virüsü (IPNV) in vivo ve in vitro replikasyonu üzerine etkilerini belirlemek amacı ile bir dizi deney gerçekleştirildi. In vitro olarak, değişik alt-gruplara ait çeşitli seviyelerde virulense sahip 109 IPNV izolatı, besiyeri içerisinde % 1 GAS içeren ve içermeyen rainbow trout gonad (RTG-2) hücresinde replikasyona tabi tutuldu. Aynı zamanda dere alabalığı (*Salvelinus fontinalis*); 1 % GAS içeren ve GAS içermeyen besiyeri ile 11 kere tekrar tekrar replikasyona tabi tutulmuş dört virülent IPNV izolatları ile enfekte edildi. Virülensi olmayan IPNV izolatlarının RTG-2 hücrelerinde RTS ile replikasyonları önlenirken, yüksek virülense sahip izolatların GAS'a dirençli oldukları görülmüştür. Dahası, in vivo olarak replikasyonun engellenme seviyesinin, izolatın virülenslik derecesi ile ters orantılı olduğu saptanmıştır ($P < 0,0001$). GAS'ın IPNV replikasyonunu in vivo ve in vitro şartlarda önlemesi virüsün serotipine bağlı olmadığı gibi aynı alt grouptaki IPNV izolatları replikasyonunda bile farlılıklara sebep olabileceği belirlenmiştir.

Anahtar Sözcükler: IPNV, 6S, replikasyon, gökkuşağı alabalık serumu, GAS

Introduction

There are studies reporting that the serum taken from normal rainbow trout that have not experienced IPN disease caused inactivation of cell culture adapted virus (CCA) passed 11 times in the presence and absence of rainbow trout serum (RTS) (1,2). Inhibition was due to an antibody-like non-virus-induced protein having 6S sedimentation coefficient. It was different from IgM having 16S sedimentation coefficient. Some, considering the effect of RTS, suggested that growth of an IPNV isolate in the presence of RTS is an important aspect of virus pathogenesis and must be considered in infection trials (3,4).

In vitro, there is variability in propagation of 6S resistant and susceptible isolates of infectious pancreatic necrosis virus (IPNV). Adsorption of Sp subtype IPNV to Fathead minnow (FHM) cells could be prevented by 1-2% rainbow trout serum (5). After incubation of FHM cells with virus in the presence of 1.3% RTS for 120 minutes, about 97% of virus remained not adsorbed to FHM cells, whereas in the absence of RTS 45% virus was adsorbed to FHM cells.

The effects of RTS on virulence of IPNV in vivo are not clear as well. Some studies indicate that virulence is correlated with serum inhibition in vitro. The virulent Sp strain of IPNV when passaged in the absence of trout

serum became avirulent (2). This avirulent virus, originally resistant to inactivation by serum, developed sensitivity to inactivation by RTS. In another study, one virulent and three avirulent strains of IPNV were passed in rainbow trout gonad (RTG-2) cells in the presence (passed 10 times) or absence (passed 10 times) of RTS (6). Originally avirulent isolates passed in the absence of RTS remained avirulent, but passage of these avirulent strains in the presence of RTS increased their virulence. However, specific data were not provided in this study. Hill (4) performed further work on 6S serum inhibition of IPNV. Three high passages of virus strains that were 6S sensitive and avirulent for fry were passed in the presence of RTS. Two 6S sensitive viruses developed 6S resistance and virulence, and the third avirulent strain remained avirulent, but developed 6S resistance indicating that 6S resistance does not always correlate with virulence. In short, some confusion exists on which serogroup or serotypes of IPNV are responsive to RTS.

In this study, unlike earlier studies mentioned above, considerable more IPNV isolates, belonging to various serogroups and types isolated from different hosts, were proposed to use to investigate inhibition of IPNV by RTS in virus propagation and cytopathic effect (CPE) formation on RTG-2 cells. Moreover, four highly virulent isolates were selected to pass in vitro in the presence and absence of RTS and then used to challenge brook trout to determine whether passing caused any the change in the level of their virulence.

Materials and Methods

Single passage in vitro experiment:

Serum:

Serum was pooled from IPNV free steelhead trout weighing approximately 50 g, obtained from Alsea hatchery, OR. Each pool included blood from at least 12 fish. Fish in this hatchery had been tested annually and no IPNV was detected within the last 15 years. After allowing to clot for 24 hours at 4 °C, the blood was centrifuged (1000 x g 20min) to separate blood cells from the serum. Aliquots were taken and stored at –80 °C (5). The serum pools were checked for toxicity on RTG-2 (7) and 1% RTS-MEM was prepared.

IPNV isolates: The IPNV isolates used were available at Laboratory for Fish Disease Research at Hatfield Marine Science Center, Newport, OR, USA. The isolates were stored at –80 °C, or in the liquid nitrogen. The isolates were obtained from various fish species, geographic areas and belonged to several serotypes.

Methods:

The RTG-2 cell line was used for virus propagation in the presence and absence of RTS. For the culture of this cell line, protocols described by Lannan (8) were followed. Eight wells of RTG-2 cell lines were prepared as two columns on each 24-well plate. One column was used for virus propagation in the presence of RTS and the other one in the absence of RTS. The virus that was used for serum sensitivity test was diluted in MEM-0 with 1% RTS. One-tenth ml of 10^5 $TCID_{50}$/ml was added to each replicate. After incubating for 1-2 hours, the RTG-2 cell monolayers were washed three times with MEM-0 and 1 ml of MEM-10 with 1% RTS was added into each of four virus inoculated wells. These eight wells were also checked everyday for 7 days during incubation at 18 °C in CO_2 incubator. Uninoculated controls were also prepared using 1% RTS in MEM-10. The same treatments, such as washing three times and using media from the same stock, were applied onto the cell monolayers on control plate. On the last day, aliquots were taken for titration and the wells were preserved with 10% formalin.

Multiple passage in vivo experiment:

Four highly virulent Buhl-IPNV isolates were used in this experiment. They were passed 11 times in the presence or absence of RTS on RTG-2 cell monolayers in 25 cm^2 tissue culture flasks.

Experimental Design

Three to four day-old RTG-2 cell monolayers, prepared in 25 cm^2 tissue culture flasks, were exposed to virus. Dilutions of each isolate, belonging to A1 serotype and Buhl subtype, with and without RTS were prepared in MEM-0. One ml of each virus dilution was added into each of the corresponding flasks. After incubating for 1-2 h, virus suspension was decanted and cell monolayers were washed three times with MEM-0. Then, 5 ml of MEM-10 with and without RTS were added into each corresponding flask. Flasks were incubated at 18 °C until at least 75% cell monolayer destruction occurred. After that, each was harvested, diluted to 10^{-1} and transferred onto new RTG-2 cell monolayers. Finally, after passing 11 times in the presence and absence of RTS, aliquots

were taken, and put at -80 °C to use in in vivo challenge experiments.

Fish

Brook trout (*Salvelinus fontinalis*), 0.9 g, obtained from Fall River Hatchery, Oregon, USA, were held in 1-l tanks at the Laboratory for Fish Disease Research at the Mark O. Hatfield Marine Science Center, Newport, Oregon. This laboratory provides dechlorinated and filtered Newport city water. Effluents in experiments are kept in a closed system and prechlorinated with 5 µg/l chlorine. Prior to release to the Yaquina Bay, it was rechlorinated with 3-4 µg/l chlorine for 2 h.

Experimental Design:

Approximately 50 fish were randomly placed into each of 1-l tank and acclimated for 5 days. Two replicates for each treatment were used. Enough virus was put into each tank to yield 10^4 $TCID_{50}$/ml. Water was aerated during 5 h exposure time.

Fish were fed and dead fish were recorded, and removed twice a day to process for titration. If there were more than five fish dead, fish were grouped into five fish pools and titered. For the first five days, mortalities were recorded and titered daily. Subsequently five dead fish pooled and titered every three days, until the end of the experiment.

Visceral organs were taken for viral sampling. After diluting 10^{-1} with MEM, samples were homogenized using a "stomacher" (Colworth, Cincinnati, USA) and assayed in 96-well microtiter plates seeded with barely confluent monolayers of CHSE-214.

Results

In vitro experiment results:

A striking relationship was observed with CPE differences in some isolates replicated in the presence of RTS (chi-square = 21.54; df = 4; P = 0.002) (Figure 1). High CPE differences coincided with low degrees of virulence. The isolates having more than 50% virulence were resistant to RTS, whereas the isolates that had low virulence or no virulence were inhibited effectively by 1% RTS. Another strong relationship ($F_{(1,65)}$ = 31.970, P ≤ 0.0001) between log_{10} titer differences on RTG-2 cells from the virus treated with 1% RTS and the virus which had no treatment and virulence of virus was also observed. When the inhibition by RTS is high, the virulence of virus tended to be low, whereas, especially, if virus caused greater than 80% mortality, there was no inhibition. In fact, virus yield in the presence of RTS increased in some Buhl subtype isolates having nearly 100% virulence. Viruses inducing moderate virulence levels (50-80%) were very variable in terms of being inhibited by RTS. Most of the isolates fit the model of that there exists a negative correlation between inhibition by RTS and virulence. Except for a single CAN-2 isolate and Buhl subtype isolates, all the other isolates were inhibited by RTS to some degree.

In vivo experiment results:

An experiment was performed with 4 Buhl subtype IPNV isolates to determine if passing virus 11 times in the presence of 1% RTS had an effect on virus virulence. No virus was detected out of any of mortality in control groups. Significant mortality levels were detected in 1 g

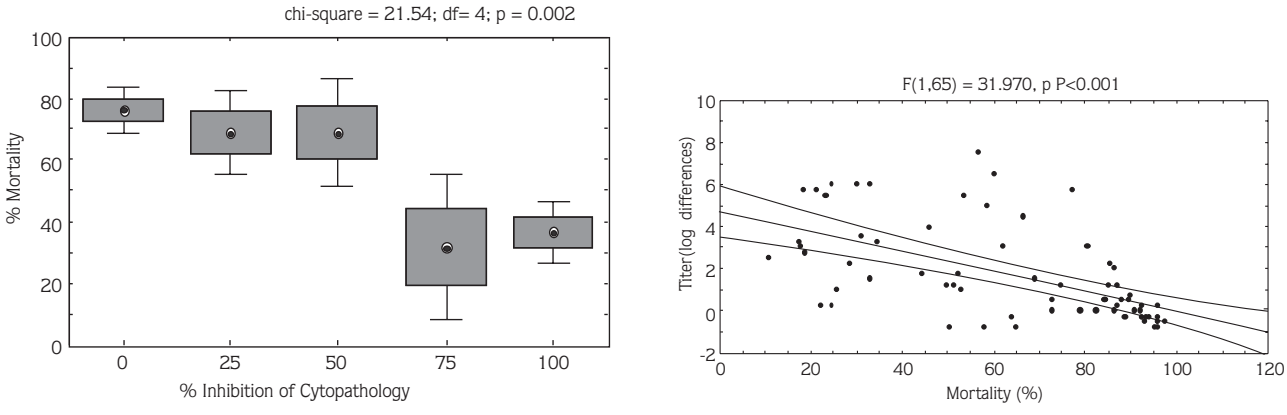

Figure 1. a) The relationship between CPE differences in RTG-2 cells infected with aquatic birnaviruses in the presence and absence of RTS and mortality levels in brook trout fry.
b) The relationship between Log_{10} titer differences in RTG-2 cells infected with aquatic birnaviruses in the presence and absence.

brook trout infected with 4 different IPNV isolates. Mortality in this size fish reached approximately 50-60% and was accompanied with behavioral and gross internal signs of IPN disease.

The repetition of virus propagation in vitro reduced virus virulence. Of two, out of 4 Buhl subtype isolates, the virus passed 11 times in vitro in the absence of 1% RTS (Data was not provided due to space limitations) lost their virulence compared to unpassaged virus. Crayfish and csf-35-85 isolates appeared to lose their virulence even in the presence of 1% RTS.

During the course of the epizootic period (5-10 days postinfection) the virus titers from moribund fish appeared higher in unpassaged and RTS-treated viruses than for high passage virus in the absence of RTS.

The 90-11 isolate passed only once produced 45% mortality and 90-11 isolate passed 11 times in the presence of 1% RTS caused approximately the same level of mortality. However, the 90-11 virus isolate passed 11 times in the absence of RTS did not produce elevated mortality. The difference in mortality level between RTS treated and untreated virus was highly significant (X^2 = 15.76, $P < 0.0001$).

Mortality in the groups exposed to H-VAT isolate was 45% both in fish infected with the H-VAT isolate passed once and the virus passed 11 times in the presence of RTS. This anticipated level of mortality were significantly higher than the control groups ($X^2 = 19.71$, $P < 0.0001$). The virus passed 11 times in the absence of RTS did not differ from controls ($z = 0.16$, $P = 0.43$) in level of mortality.

The mortality caused by the csf 035-85 isolate was significantly higher than the control groups ($X^2 = 51.77$, $P < 0.0001$). The csf-035-85 IPNV isolate passed once caused significantly higher mortality than that caused by csf-035-85 isolate having no RTS treatment during 11 times passage in vitro (ANOVA; $F_{(52,144)} = 1.74$, $P = 0.005$). On the other hand, no difference was observed between the level of mortality caused by the virus passed 11 times in the presence and the virus passed in the absence of 1% RTS ($z = 0.16$, $P = 0.43$).

No difference was detected between negative controls and the crayfish isolate viruses passed 11 times in the presence or absence of RTS ($X^2 = 6.17$, $P = 0.46$), whereas the virus passed once proved to be virulent to brook trout.

Titers: The titers obtained in the exposure of trout to the 90-11 isolate passed in the presence or absence of RTS were significantly different from each other (ANOVA; $F_{(25,2)} = 3.94$, $P = 0.032$). In all other three trials, passing with or without RTS did not show any statistical difference ($P > 0.05$) in mortality levels in trout.

Discussion

Studies with radioisotope-labeled virus showed that inactivation of IPNV by RTS is due to inhibiting of binding to the host cell surface (5) but it occurs prior to attachment. This inhibition is not due to induction of interferon or binding to cellular receptors, and it does not depend on some cell variants, the viruses obtained by passing in different cell lines, like FHM-IPNV or RTG-2-IPNV. Overall serum titers in CHSE-214 and FHM cells were low but identical showing that serum activity was directed against FHM-IPNV or RTG-2 IPNV variants.

Avirulent isolates of IPNV were inhibited from replication on RTG-2 cells even after a single passage in the presence of RTS. These results supported those of Vestegard Jorgensen (1), Dorson et al. (2) and Dorson et al. (3). It was not possible to test RTS inhibition on FHM cells because of cellular toxicity of 1% RTS on this cell line. However, since, as reported by Kelly and Nielsen (5), RTS acts prior to attachment to cells, host cell origin differences should not affect susceptibility to RTS in vitro. Host range might be important in observing development of 6S sensitivity relative to virulence alterations with passage level. For example, serum sensitivity developed faster in EPC cells than RTG-2 and BF cells (6). The results that will be obtained from this kind of study will help to establish standard procedures to propagate virus for infection trials and immunization.

The results here supports the findings of Kelly and Nielsen (5) reporting that serum inhibition is not dependent on the virus serotype. Buhl subtypes having the highest virulence values had the lowest inhibition of replication among the IPNV isolates tested. WB, Jasper, Ab, Sp, Te, BC and He subtypes were inhibited the most since most of the isolates included within these subtypes were avirulent (9). For example, the 93-321 a virulent isolate belonging to WB subtype was not inhibited ($\log_{10}$ decrease = 0.5). On the other hand, 92-326 isolate which is also a WB subtype isolate, but was avirulent (21.5%) the titer difference was also highly significant ($\log_{10}$ = 7.2).

In short, isolates having high degree of virulence were consistently resistant to RTS as, for instance, was the case in Buhl subtype isolates. It is suggested to propagate virus in the presence of RTS for infection. Before challenge trials, it is important to confirm that the virus is not 6S sensitive. If it is 6S sensitive, it will be neutralized by RTS effectively.

The number of in vitro passages is known to influence virus virulence. IPNV loses its virulence to some degree through in vitro passage. However, fewer than five passages do not significantly alter virulence of the virus (3,6). Similarly, the Sp isolate passed 5 times produced 50 % mortality, whereas at the 11^{th} passage, it caused only 9% mortality (10). This isolate at the original isolation had caused 90% virulence in trout (1). Similarly, the VR 299 isolate, which caused 90% mortality originally, passed 5 times in the absence of RTS produced 20% mortality, and in the presence of RTS, 50% mortality was observed (11). At 15^{th} passage this isolate was avirulent regardless of the presence of serum. My findings were also supportive of results indicating loss of virulence by multiple passages. In four out of four cases, viruses passed 11 times in the absence of RTS lost their virulence. In two out of four cases, virus isolates, 90-11 and H-VAT, passed 11 times in the presence of RTS retained their virulence, whereas the other two isolates, crayfish and 35-85, lost their virulence although they were passed in the presence of RTS. This strongly suggested that these viruses became avirulent after passing 11 times despite the presence of 1% RTS in the culture medium. These findings were supportive of McAllister and Owens (11) reporting that virulence was not preserved by the presence of RTS during virus propagation. In all four cases, mortalities from the isolates that passed only once were the highest, suggesting that virus passage in the presence of absence of RTS affects virulence of virus.

The effects of RTS on preservation of virulence during multiple passage remains in question. Hill reported that virulence of aquatic birnaviruses could be preserved by using RTS during virus propagation on cell monolayers (4). Moreover, he added that avirulent isolates could be made virulent by multiple passing in the presence of RTS. However, the avirulent isolates made virulent did not induce antibody production whereas naturally virulent isolates induce it. No specific data were provided in Hill's report (4) or in the later report by Hill and Dixon (6) on this aspect. In contrast, it was reported that IPNV virulence could not be preserved using RTS and FBS must be used instead (11). According to the data provided, the virus passed in the presence of 5% FBS and the virus passed in the presence of 5% RTS both lost their virulence entirely at the passage number 15. One drawback to this study was that only one isolate, VR 299, was used. Even though no difference was detected, fetal bovine serum was ironically suggested to use in virus propagations.

In terms of preservation of virulence the results obtained in this study do not agree with the statements made by both Hill (4) and McAllister and Owens (11). In some cases virulence can be preserved, though it is not always the case as suggested by Dorson et al. (3). In two out of four trials, virulence was preserved suggesting that virulence could be preserved using RTS, whereas the other two Buhl subtype isolates lost their virulence after passing 11 times in the presence of RTS. It is highly possible that there may be some other factors related to viral mutation playing role in resistance to RTS. Some alterations in viral components that are important in the determination of virulence may be preserved for some passage number by RTS during cloning.

In conclusion, the effect of RTS on virulence is variable, even within the same subtype of IPNV (Buhl). There is evidence for some kind of effect of RTS on preservation of virulence, but variability of effects on IPNV isolates remains unknown. Especially, for challenge experiments with IPNV, usage of RTS should be standard in culture medium.

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