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Characterization of humoral immune response to pancreas disease virus (SAV3) following immunization with whole inactivated virus vaccines and DNA vaccines

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Master in Aquaculture

Norwegian University of Life Sciences (NMBU), Ås

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Master of Science (MSc) THESIS

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Muhammad Mujahid Amjed

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APC	Antigen-presenting cells
CHH1	Chum salmon heart cell 1
CHSE-214	Chinook salmon embryo-214
CPE	Cytopathogenic effect
CMS	Cardiomyopathy syndrome
DPA	Docosapentaenoic acid
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DNAV	Deoxyribonucleic acid- vaccine
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
FBS	Fetal bovine serum
HSMI	Heart and skeletal muscle inflammation
HRP	Horse radish peroxidase
HCL	Hydrogen chloride
IWA	Inactivated whole virus vaccines
IFAT	Indirect fluorescent antibody test
IPN	Infectious pancreatic necrosis
ISA	Infectious salmon anemia
IHN	Infectious hemopoietic necrosis
IgD	Immunoglobulin D
IgM	Immunoglobulin M
IgT	Immunoglobulin T
mu-IWA	multivalent inactivated whole virus vaccine
mo-IWA	monovalent commercial inactivated
OD	Optical density
OAV	Oil adjuvanted vaccines
PDV	Pancreas disease virus
PRR	Pattern receptors recognition
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline containing 0.5ml/L Tween-20 (PBST-20)
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SAV-3	Salmonid alphavirus type 3
SPDV	Salmon pancreas disease virus
SD	Sleeping disease
STATA	Statistical software for data science
$TCID_{50}$	tissue culture infective $dose_{50}$
TMB	3,3',5,5'-Tetramethylbenzidine
TRIM-21	Tripartite Motif
Trypsin-EDTA	Trypsin- ethylenediaminetetraacetic acid
VN	Virus neutralization

ABSTRACT

Salmonid alphavirus type-3 (SAV-3) is the causative agent of pancreas disease (PD) in Atlantic salmon. It is associated with significant economic losses to the aquaculture industry. Vaccines can overcome losses through vaccination. In this study, anti-SAV-3 antibodies in plasma samples from Atlantic salmon vaccinated with DNA, inactivated whole virus vaccines (IWV) and combined delivery of were quantified through indirect enzyme-linked immunosorbent assay (ELISA). Plasma samples at 1:50 dilution was found to have neutralizing antibodies using 100 $TCID_{50}$ SAV-3, but additional testing showed no neutralization at 1:20, 1:40, 1:80, and 1:160 dilution of plasma samples tested on both chinook salmon embryo (CHSE-214) and chum salmon heart (CHH-1) cell lines. At 1:20 dilution, cytotoxicity was observed in CHSE-214 and CHH-1 cell lines. Furthermore, there was no significant effect of complement on neutralization, although a tendency towards delayed CPE was observed. In summary, immunization of Atlantic salmon with plasmid-encoded (DNA), inactivated whole virus vaccines or combined delivery of intraperitoneal and intranasal induced low levels of circulating antibodies tested by ELISA. Classical virus neutralization assays gave very low to no neutralizing titer. However, employing an indirect fluorescent antibody test (IFAT), titers were found to be 40-80 with some variation between vaccines while for some fish in the DNA vaccinated group, above 80 (no endpoint dilution obtained). It is concluded that an ELISA and IFAT-based neutralization assay can be combined to estimate and document the presence of anti-SAV-3 antibodies post vaccination but correlation with protection against disease is not known.

Keywords: Atlantic salmon, SAV-3, CHSE-214, CHH-1, Indirect ELISA, Neutralization, Antibody, Complement, Dilutions, $TCID_{50,}$ Vaccines

INTRODUCTION

AQUACULTURE

Aquaculture evolved in Asia as a freshwater food system and has now turned into industrial production across the globe. This industry now guarantees food security for a growing population and comprises almost 45% of world food fish consumption (FAO, 2006). The Food and Agricultural Organization (FAO) of the United Nations (UN) estimates that the global population will grow to 9.74 billion by 2050. This increase in population will be accompanied by the need for more food, and a sustainable aquaculture industry can potentially help in fulfilling the requirement (Frankic & Hershner, 2003). Aquaculture production in 2019 was 85.3 million tons and the total sale value was estimated to be 260 billion US dollars (USD) (Veterinærinstituttet, 2022). The global per capita consumption of fish slightly increased from 20.3 kg in 2017 to 20.5 per kg in 2019**.** The expected growth is an annual growth of 12% during 2020–2029 with an estimated world population growth of 9%. By 2029, per capita, fish consumption is estimated to be 21.4 kg while it was 9.9 kg in the 1960s and 20.6 kg in 2020. This is equivalent to another twenty million tons of seafood supply, which aquaculture is estimated to provide. Latin America and Asia are expected to have the highest growth, while negative growth is expected in Africa. In general, per capita, fish consumption is likely to grow faster in developing countries. However, more developed economies are expected to have the highest per capita consumption (*MOWI: Integrated Annual Report 2021*).

SALMON AQUACULTURE

Atlantic salmon is an anadromous fish species belonging to the family *Salmonidae* and is a popular seafood worldwide. Norway, Chile, Scotland, and Canada produce more than 90% of the global production . Nowadays about 80% of the world's Salmon harvest is farmed. Salmon consumption is considered healthy due to its high protein and omega-3 fatty acids (including eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA)), minerals, and vitamins. In 2013, Atlantic salmon became the most important commodity traded in terms of value and accounted for about 18 percent of the total value of internationally traded fish products in 2019 Since the 1970s, initiatives in salmon farming have revolutionized the salmon industry and the whole seafood industry both in Norway and worldwide (Hjelt, 2000; Sønvisen, 2003). In a few

decades, Norway has grown to become the world's largest farmed salmon producer, and farmed salmon is now the fourth biggest export commodity in Norway.

CHALLENGES FACED BY THE AQUACULTURE INDUSTRY

The aquaculture industry is facing several social, environmental, economic, and governmental sustainability challenges. Sustainable production is linked to lower carbon emissions, sustainable feed ingredients, and proper management practices (Boyd et al., 2020). Fish health and welfare are at the center of all these challenges and are important for the success of future expansion plans (Toni et al., 2019). Moreover, infectious diseases are one of the major causes of biological and economical loss in aquaculture across the globe (Macqueen et al., 2021; Weston et al., 2002; Pettersen et al., 2015; Veterinærinstituttet, 2020, Herath and Thompson 2022). Pancreas disease, caused by an infection with salmon pancreas disease virus (SPDV), also termed salmonid alphavirus (SAV) constitutes a big constraint for the farmed salmon industry in Europe (Jarungsriapisit et al., 2016).

Several bacterial, viral, and parasitic diseases have been affecting the farmed Atlantic salmon industry. However, most bacterial diseases have been controlled after the introduction of vaccines (Håstein et al., 2005; Pridgeon & Klesius, 2012). Viral infections on the other hand have continued to cause economic losses for industry. Common viral diseases affecting the salmon industry include pancreas disease (PD), infectious pancreatic necrosis (IPN), cardiomyopathy syndrome (CMS), infectious hemopoietic necrosis (IHN), infectious salmon anemia (ISA), and heart and heart and skeletal muscle inflammation (HSMI). PD has been one of the main viral diseases affecting the salmon industry Norway in recent years.

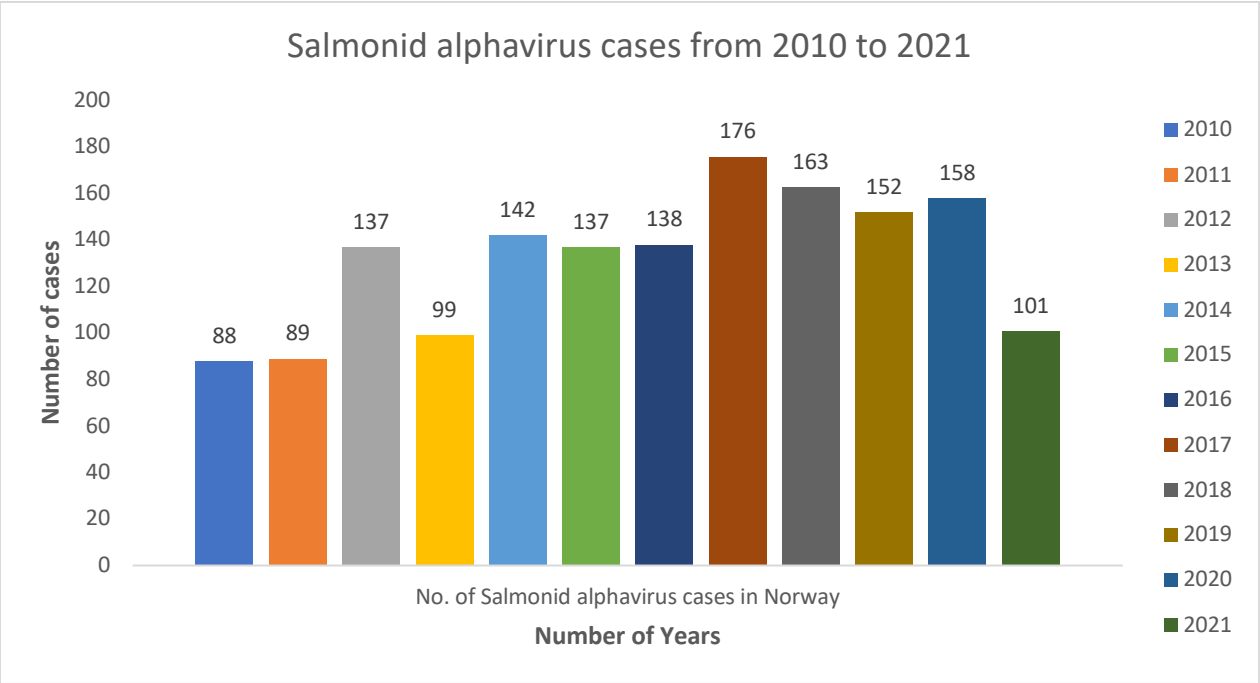


Figure 1. Graphical representation of the number of Salmonid alphavirus (SAV-3) cases from 2010 to 2021 (Veterinærinstituttet, 2022)

The numbers of PD-registered outbreaks are presented in Figure 1. A general increasing trend in the reported cases is observed up to 2019, with the number of cases increasing from 88 in 2010 to 158 in 2020. The highest number of outbreaks were observed in 2017 (176) and 2018 (163) while a drop in the reported cases to 101 was observed in 2021. Since 2014, PD has been listed by the world health organization for animal health (OIE) as a notifiable (national list 3) disease in Norway. In 2020, PD was among Norway's three dominating diseases affecting salmonid aquaculture. PD was ranked 8th place as a cause of mortality in growing salmon and ranked first in causing retarded growth (Sommerset et al., 2022)

PANCREAS DISEASE (PD)

Pancreas disease (PD) was first reported from Scotland in 1984 (Munro et al., 1984). Later, it has been detected in Western Europe and North America (Weston et al., 1999). It is a highly contagious and endemic viral disease in wild and farmed Atlantic salmon (*Salmo salar* L.) and can affect the fish during the entire marine production cycle. The severity of the disease is less in rainbow trout as compared to Atlantic salmon. Despite vaccination, the number of reported cases is still high.

The causative agent is salmonid alphavirus (SAV), a single-stranded positive-sense ribonucleic acid (RNA) virus belonging to the family *Togaviridae*. It is the only Alphavirus for which fish is the natural host (Powers et al., 2001). SAV has been categorized into six subtypes SAV1-SAV6 SAV1, SAV2, SAV4, SAV5, and SAV6 have been reported from farmed Atlantic salmon and rainbow trout around the British Isles (Herath & Thompson, 2022). SAV2 infection causes sleeping disease (SD) in rainbow trout. Different subtypes are genetically similar and related isolates of the same virus species (Weston et al., 2002).

SAV-3 is found in western Norway south of Trøndelag county while SAV2 is the dominant strain in central Norway (Trøndelag). Recently, a seventh genotype, SAV7, has been identified in the Ballan Wrasse in Ireland (Teige et al., 2020). The virus genome (Fig.2A) size is 11.9 kilobase pairs (kb) and contains two large open reading frames which encode four non-structural proteins (P_1, P_2, P_3, and P_4) and four structural proteins (E1, E2, E3, and 6K/TF) constituting the spherical icosahedral-shaped (Fig. 2B) virus particle (Woo & Cipriano, 2017)

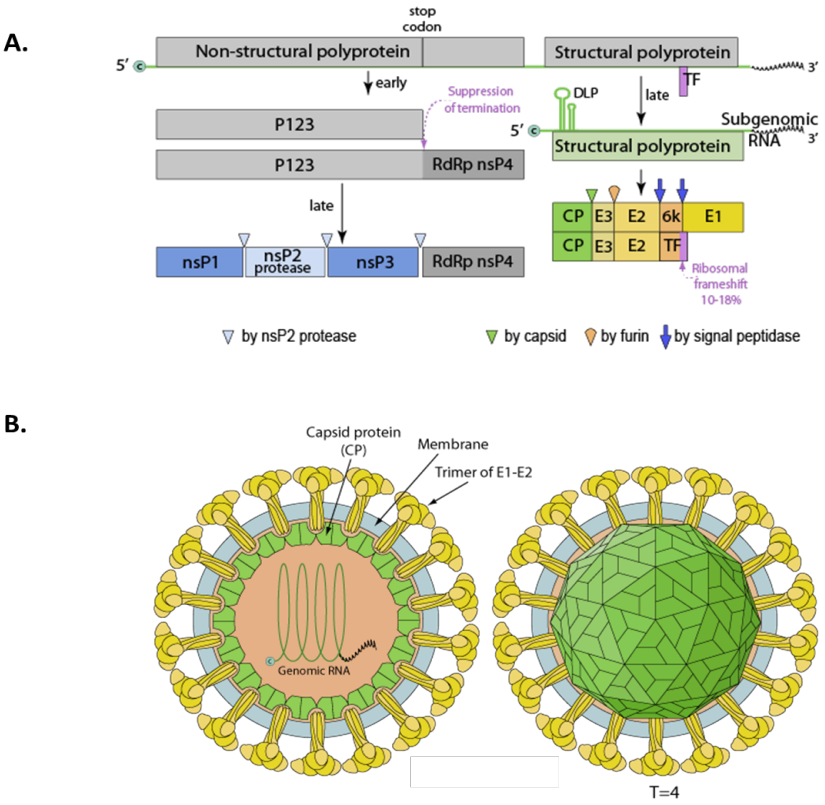


Figure 2. The genomic and virion structure of alphaviruses. A. Illustration of the different ORFs and the encoding proteins. B. The virion structure shows the enveloped, spherical icosahedral capsid with a T=4 icosahedral symmetry. The envelope contains 80

spikes, and each spike represents a trimer of E1/E2 proteins. (Source: Viral Zone: https://viralzone.expasy.org/625?outline=all_by_species)

The cell membrane of the host cells is flanked by the inner part of the virion capsid. The membrane is penetrated by the main antigenic target glycoprotein E1 (Karlsen et al., 2015; Moriette et al., 2005; Voss et al., 2010) which, together with E2, is the main antigenic target for the immune system (Moriette et al., 2005). E2 is the receptor-binding protein of SAV (Villoing et al., 2000). The poorly studied features like filopodial membrane projections, cytoplasmic stress granules, and putative intracytoplasmic budding have been suggested to play important role in disease persistence (Noguera et al., 2021). The entry route is not precisely defined but the intestine, gills and skin are considered likely entry routes (McLoughlin et al., 1996).

EPIDEMIOLOGY OF PANCREAS DISEASE (PD)

The epidemiological studies showed that the virus is circulating all year round on the Norwegian coast but the high rise in water temperature in summer (10 ^{0}C-15 ^{0}C) is the main stimulating factor for outbreaks (McLoughlin et al., 2003). In addition, the stress generated during fish handling and mechanical delousing at marine production sites can also be a triggering factor.

Initially, the infected fish exhibits reduced feed intake, gathers at the water surface in the pen, becomes sluggish, and sometimes dorsal fins become visible above the water surface. This can initially be observed in one pen that eventually spreads to adjacent areas. The disease can be classified into per-acute (0-4days), acute (5-10 days), sub-acute (11-22 days) chronic (22-42 days), and recovery (43 days) phases. The distinct histopathological lesions include loss of the exocrine pancreatic tissue initially followed by inflammation of the heart and later of the skeletal muscle, both red and white muscle. The outcome of the disease is impaired growth, reduced fillet quality, reduced welfare, and a relatively high mortality rate. Infected salmon can become carriers and shed the virus for a long period even with negative screening tests (Veterinærinstituttet, 2020).

During the viremic phase, the infected fish shed the virus to the adjacent areas and the surrounding fish population becomes infected. In farmed fish, horizontal transmission through the exchange of equipment and transport of fish has been reported. However, it is generally still unclear how farmed salmon become infected, but it is thought that wild fish species such as long rough dab

(*Hippoglossoides plates soides*), common dab (*Limanda limanda*), and the plaice (*Pleuro nectes platessa*) are wild reservoir that can be the source of initial infection. Salmon lice have also been proposed as a vector since viral RNA can be detected in the lice (Karlsen et al., 2006) (Petterson et al., 2009).

So far, SAV-3 has only been reported from Norway while SAV2, with two distinct lineages, causes disease in Norway, Scotland, and other European countries. In Norway, recent outbreaks of mixed infection involving SAV-3 and SAV2 have been reported in 2020. An increasing trend in SAV-3 infections was observed from 98 in 2019 to 110 in 2020, with a reduced number of clinical cases being reported in 2021. Based on epidemiological data, the west coast of Norway has been divided into three regions as non-endemic, and SAV2- and SAV-3-endemic zones (Fig. 3 Macqueen et al. 2021).

The first PD legislation was introduced in 2007 by the Norwegian authorities which made PD a nationally notifiable disease (list 3) and established national regulations as to how to handle the disease. The legislation was further modified in 2017 (2017-08-29, nr.1318) to curtail the spread of that infection along the long Norwegian coastline. The number of registered cases has significantly dropped from 2016 to 2021 to 176 in 2017, 163 in 2018, 152 in 2019, 158 in 2020, and 101 with 82 confirmed cases in 2021 (Madhun et al., 2022).

PD has been associated with significant economic losses and PD is known to be one of the main causes of production losses for industry. On the contrary, little data is available about losses caused by sleeping disease (SD) because the disease affects smaller-sized rainbow Trout and is not reported properly and continuously.

Figure 3. The current definition of SAV endemic and non-endemic zones (Macqueen et al., 2021).

Early harvesting of biomass, a PD preventive measure

A developed bioeconomic model was compared for the salmon biomass undergoing pre-scheduled harvesting versus salmon biomass going through PD outbreaks. The model was based on data from national statistics, production companies, expert committees, and stochastic simulations. Polymerase chain reaction (PCR) was used for virus detection in the salmon biomass and indication for early harvesting. They recommended that the bio-economic model is beneficial with pre-scheduled harvesting at an average salmon weight of 3.2 kg comprising 1000000 (1×10^6) smolts. Direct loss of 55.4 million Norwegian Kroner (NOK) was predicted in nine months by the PD outbreak. High sales prices substantially contributed to the losses linked with the PD outbreak in 2013 because losses were sensitive to the feed-salmon sales price. PD in Norwegian, Scottish, and Irish aquaculture is

a source of loss in terms of reduced gross weight, reduced fillet quality sharply increased mortality, and increased feed utilization (Pettersen et al., 2015).

IMMUNE RESPONSE

The immune system consists of a complex network of innate and adaptive immune cells and molecules that are used to protect the body from invading pathogens. When effective vaccines are not available then there is a need for a comprehensive investigation of virus-host interactions to understand the effective immune responses required for protection in order to develop new control strategies (Asha et al., 2021). Different viruses have developed mechanisms to survive and escape from the innate immune system, for example through evading the recognition by the pattern receptors (PRR) which are used to detect specific microbial molecules ((Faure & Rabourdin-Combe, 2011). The viral evasion strategies are variable and may consequently result in initiating an incomplete, delayed, diminished, or strong immune response. Based on the type of response generated, the activated immune responses may lead to persistent infection and damage to the host (Kikkert, 2020). The strategies employed by SAV include impairment and evasion of host immune system activity by altering cytokine secretion, affecting interferon release, decreasing antigen presentation, and interrupting cell death mechanisms (Dahle & Jørgensen, 2019).

INNATE AND ADAPTIVE IMMUNE RESPONSES IN HIGHER VERTEBRATES

The immune system of higher vertebrates comprises a diverse set of physical, humoral, and cellular factors (Fig. 4) that are used to protect the body from invading microorganisms, classified into innate and adaptive immune components. While the innate immune system relies on the germ line encoded receptors for pathogen recognition, the adaptive immune system is based on somatically produced antigens receptors. Two kinds of lymphocytes T cells and B cells play a significant role in the adaptive immune system (Medzhitov & Janeway Jr, 1998). Specific memory is an integral part of the adaptive immune system of higher vertebrates, but studies revealed that the innate immune system of invertebrates and vertebrates are primed through infections for later improved reactions to similar pathogens (Kurtz, 2004), also referred to as immune training. Upon exposure to invading pathogens, innate immune responses are stimulated, and diverse responses are generated. This will eventually lead to the development of different types of inflammatory responses. Cytokines and other humoral factors constitute the early responses that prime

subsequent responses where macrophages and dendritic cells are key effector cells involved in producing these cytokines and inflammatory mediators (Fig. 5). In addition to directing the innate responses, macrophages and dendritic cells also participate in directing adaptive immune responses by processing and presenting foreign antigens to T cells (Jean-Pierre et al., 2021).

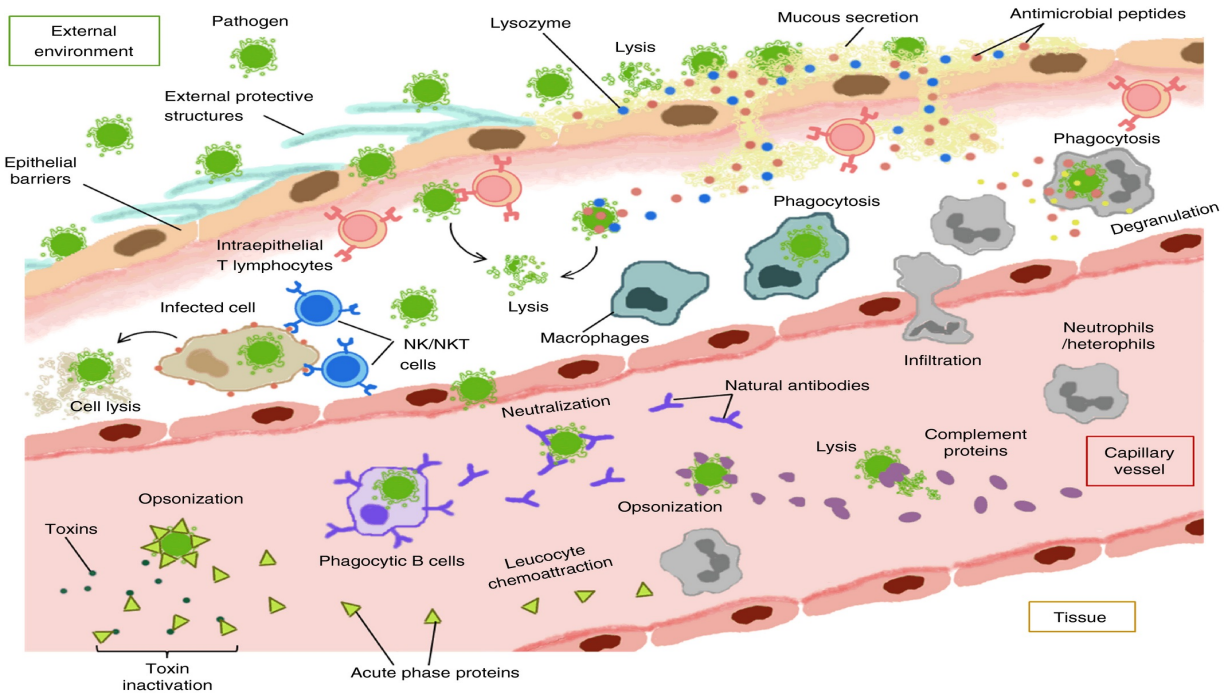


Figure 4. Schematic illustration of the main innate defense systems in jawed vertebrates, which represents its physical, humoral, and cell-mediated components and the interactions between them, in a physiological context (Riera Romo et al., 2016).

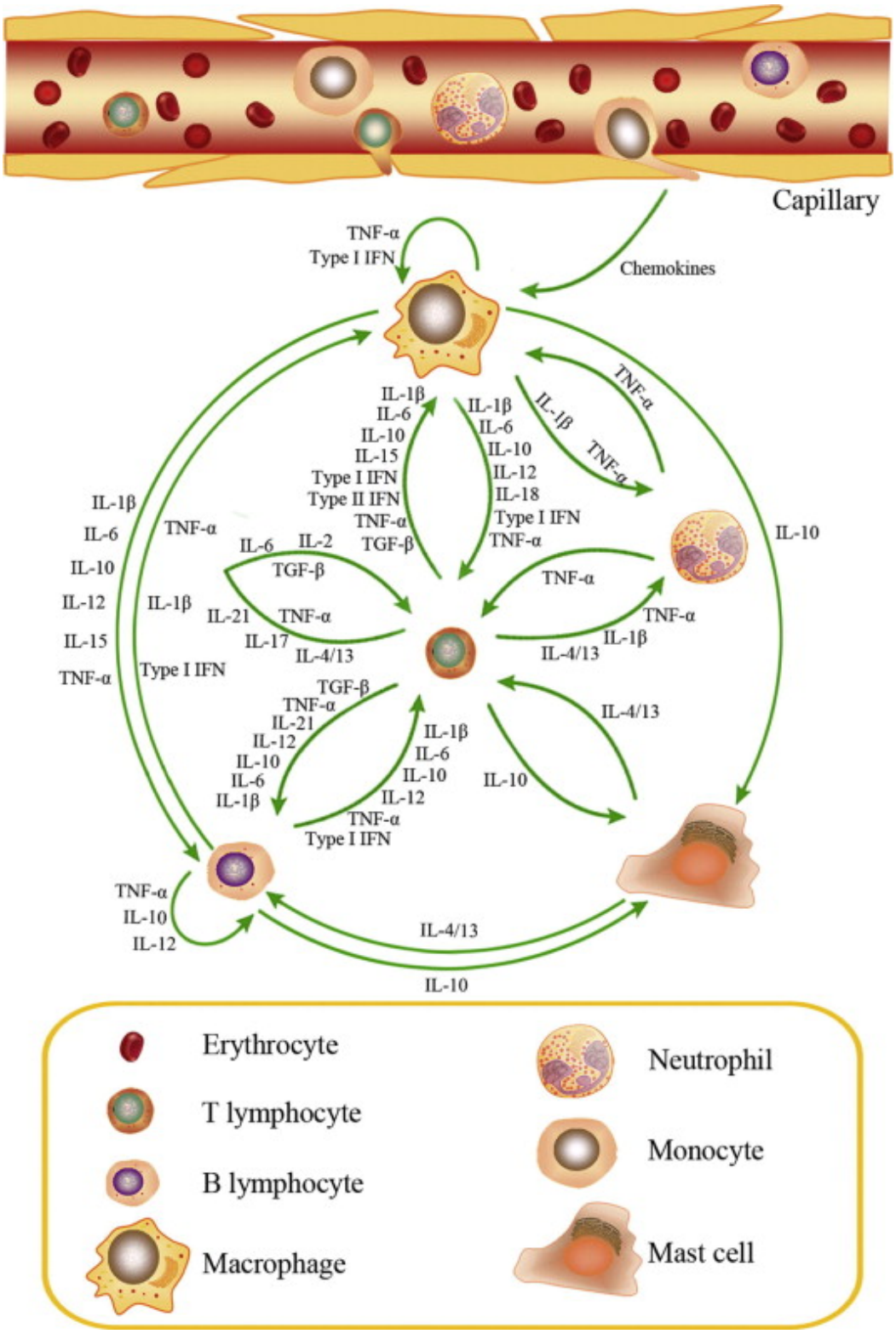


Figure 5. Schematic representation of the known cytokine network regulating inflammatory cell functions in fish, including cell proliferation, differentiation, survival or apoptosis, and numerous gene expressions (Zhu et al., 2013).

The details of the function of the fish immune system is not as well understood as in higher vertebrates (Suttle, 2005). Main differences between fish and higher vertebrates are lack of lymph nodes and the differences in the immune organs involved (Figure 6). In addition, erythrocytes are found to play a role in innate immunity in some fish species. Similar to vertebrates, however, the innate immune system comprises macrophages, neutrophils, and different non-specific cytotoxic cells. It is also well documented that adaptive immune responses are mounted when fish are exposed to pathogens or infected. Adaptive immune responses are specific but slow to develop. The major components of this system are B and T cells as in higher vertebrates. The mucosal antibodies in salmon are IgM, IgD, and IgT (Salinas et al., 2011).

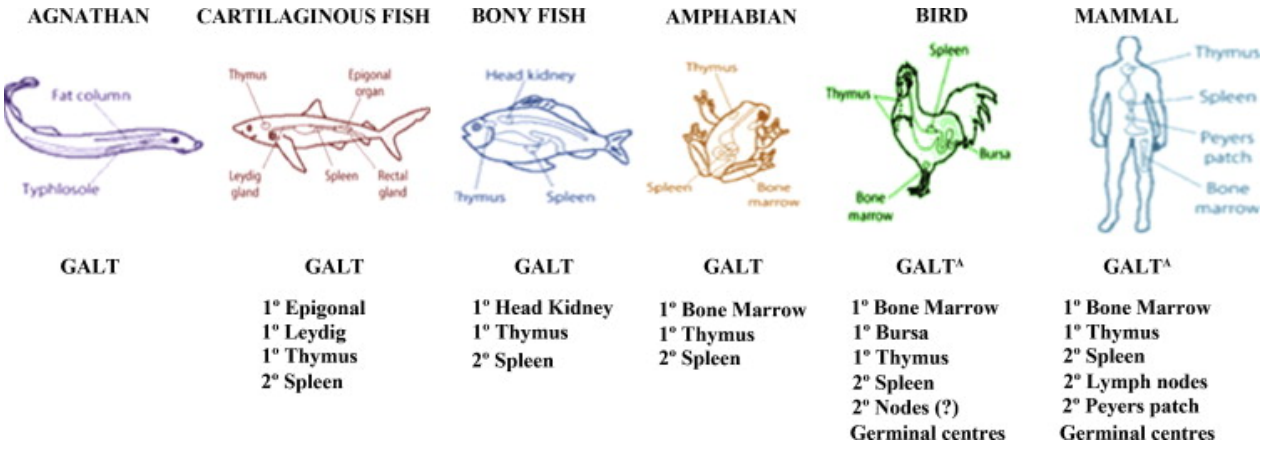


Figure 6. Immune organs in different vertebrates (Rauta et al., 2012).

ANTIBODY RESPONSES AND PROTECTION

As already mentioned, adaptive immune responses in fish are usually slow and may take several weeks to develop, particularly in cold water fish species. Despite this, the adaptive responses are effective and can successfully prevent fish from becoming infected. Dendritic cells have been characterized in the skin of fish, but their function is less described, (Alesci et al., 2020) and it is considered that macrophages and possibly B cells are the main antigen-presenting cells (APC) eliciting adaptive response (Munang'andu & Evensen, 2015). In higher vertebrates, lymphocytes are key cells responsible for the diversity of antigen recognition, specificity, and memory. These

cells are divided into B cells and T cells. B cells are responsible for antibody production and T cells are specialized for cell-mediated immunity. The T cells are further divided into cytotoxic T cells that kill the infected and abnormal cells and helper T cells that stimulate other immune cells through cytokine production. Furthermore, cytokine production constitutes the basis for the classification of the T helper cells into different types and groups (Hope & Bradley, 2021).

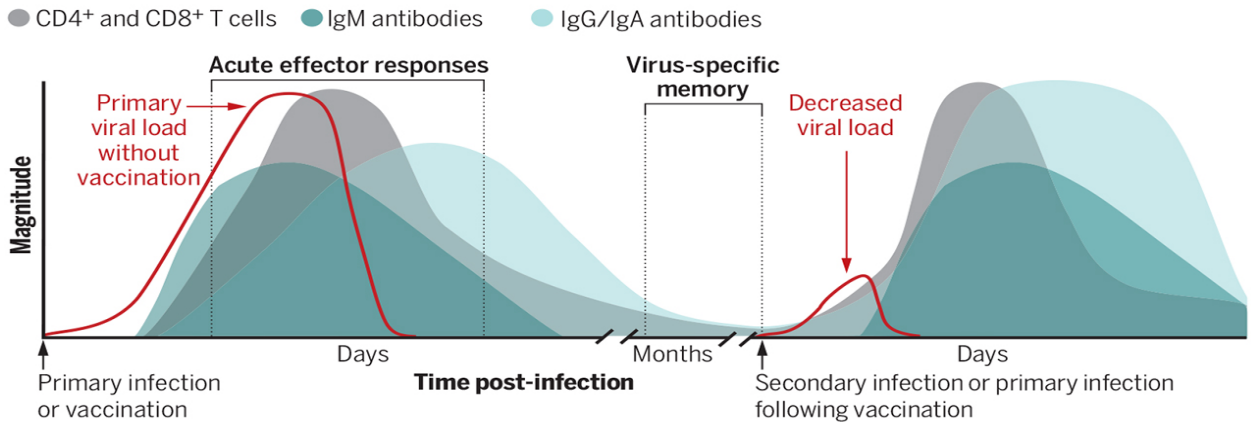


Figure 7. Adaptive immune response to viral infections – taken from higher vertebrates (Hope & Bradley, 2021)

Teleost fish have an adaptive immune system with a lot of similarity to mammalians but also with marked differences. The adaptive immune system of fish is temperature dependent and takes a longer time to develop as compared to mammals. Specific B cells produce specific antibodies but, in contrast to higher vertebrates, no class switching exists (Hordvik, 2015). Antibody isotypes are relatively limited and only three classes of antibodies have been identified thus far, namely IgM, IgD, and IgT. IgM isotype is dominant systematically and IgT is predominant in the gut mucosa and likely the skin. The function of IgD is not yet well understood (Hordvik, 2015). The presence of classical immunological memory, as defined in higher vertebrates, has not been demonstrated and only low secondary responses are thought to be generated was suggested that fish use long-lived plasma cells and low-affinity and high avidity upon repeated exposure to compensate for lack of memory B cells (Semple & Dixon, 2020).

Antibody responses generated during viral infections are an integral part of the adaptive immune system in Atlantic salmon. It was demonstrated that antibody responses play a role in protection against disease using passive immunization of salmon (Houghton & Ellis, 1996).

Neutralizing antibodies were detected in immunized fish (Xu et al., 2012) but their role is not understood in detail (Houghton & Ellis, 1996). It was recently shown that both IgM-positive and IgM-secreting B cells are increased in Atlantic salmon after peritoneal infection with SAV-3 (Jenberie et al., 2020). These findings indicate the possibility of using vaccination as an effective measure to protect against SAV infections.

COMPLEMENT SYSTEM

Complement is an essential part of the immune system and aids in innate and adaptive immune responses against viruses and other pathogens. It plays a key role in protection through enhancement in antibody neutralization and opsonization, increased phagocytosis through immune cells and elimination of the pathogen. Since viruses are intracellular "parasites", prompt elimination is through binding to virus surfaces with a primary aim to prevent systemic distribution. Cytotoxic lymphocytes play a major role in destroying infected cells. In contrast, antibodies bound to antigen will also bind complement (active the complement cascade) and plays an important role in lysis of bacteria and membrane viruses. IgM binds to surface proteins of viruses and can activate complement while this is not shown for IgT. Complement activation pathways include classical and alternative (Sissons & Oldstone, 1980), where antibodies will activate complement through the classical route.

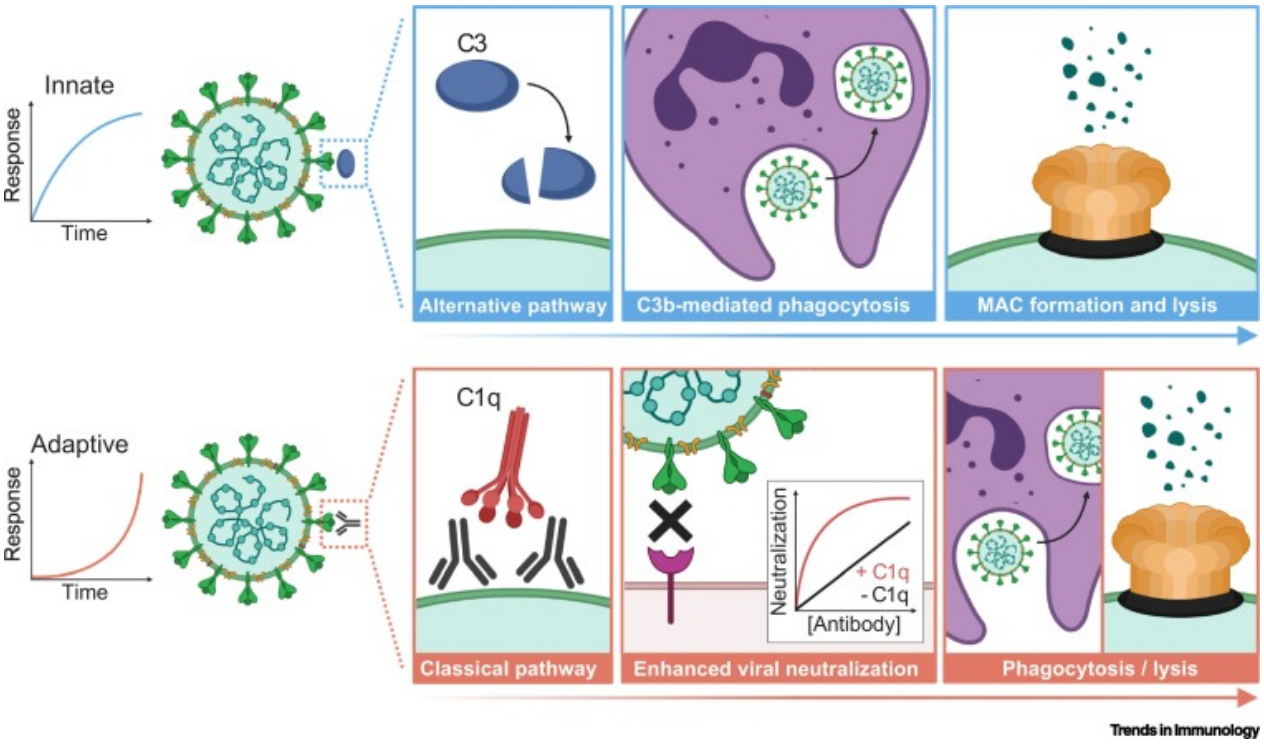


Figure 8. Potential mechanism of innate and adaptive complement activation immunity against Severe Acute Respiratory Coronavirus-2 (SARS-CoV-2), (Kurtovic & Beeson, 2021)

VACCINATION

Overall, vaccination has been proven to be a successful control measure in aquaculture (Gudding & Van Muiswinkel, 2013) and is considered one of the safest methods to protect against diseases (Cao et al., 2017). In addition to protecting individual fish, vaccination will give herd immunity and that limits the spread of microorganisms in the aquatic environment. Atlantic salmon is vaccinated at 40-60 grams and transferred to sea at 80-100 grams weight (Jensen et al., 2012). One of the earlier indications of the protective effect of vaccination of fish came in 1938 when carp were found to be protected against *Aeromonas punctata* through vaccination (Snieszko et al., 1938). Later, trout was found to be protected against *Aeromonas salmonicida* infection when vaccinated by parental inoculation and oral administration (Duff, 1939; Duff, 1942). Knowledge generated through these early studies has been effectively translated into commercial vaccines, especially for salmonids and the first vaccine for aquaculture against yersiniosis in salmonids was approved in the United States of America (USA). Consequently, several fatal bacterial diseases have been controlled through a successful vaccination in the Norwegian salmon industry (Pridgeon & Klesius, 2012). In contrast, viral vaccines are currently less efficacious, but they still have the

potential as an effective measurement to control prevalent SAV infections (Evensen & Leong, 2013).

Vaccination against pancreas disease (PD) can prevent mortality and virus shedding to neighboring marine farms. The virus is transmitted horizontally and seawater currents could be a possible cause of virus spread in the vicinity (Skjold et al., 2016). The available oil adjuvanted vaccines (OAV) succeeded in reducing economic losses from PD. Meanwhile, plasmid encoded (DNA) vaccines proved protective when tested in experimental challenge. OAVs have been compared with DNAV with normal saline as a control. The fish were injected at 10400 days at 12 °C. The experimental fish were challenged by cohabitation and the DNA vaccinated fish showed a significantly higher level of neutralizing antibodies titers, a significantly lower level of viremia, and reduced transmission to unvaccinated fish, plus significantly better weight gain (Thorarinsson et al., 2021).

The current PD vaccines have been found to decrease the number of PD outbreaks in Norway. Positive effects are seen as reduced mortality rate and the number of fish subject to downgrading at slaughter. The vaccination and infection pressure were shown to be inversely proportional to each other (Sommerset et al., 2022). This is probably due to the fact that vaccines against PD have significantly reduced the shedding of virus in infected salmon (Skjold et al., 2016). The risk of disease is decreased to about half in spring smolt due to larger weight and better immune system in favorable weather conditions (Jensen et al., 2012).

The industry is demanding a potent PD vaccine to prevent or limit disease loss. Inactivated whole virus vaccines (IWV) have been criticized for not preventing outbreaks although the number of outbreaks dropped during a period (Jensen et al. 2012). IWV and plasmid based (DNA) vaccines are now available in the market, and the first PD vaccine was licensed in 2007 (Norvax® Compact PD, Intervet International B.V.). The mortality was reduced to 50% at vaccinated farms. Pharmaq AS has manufactured a monovalent IWV, oil adjuvanted, Pharmaq Micro-1 PD and a multivalent vaccine, Pharmaq micro-6 PD. Clynav, a plasmid-based vaccine, was granted marketing authorization in the European Union (EU) in 2019. Traditional inactivated virus vaccines have potential for improvement in terms of efficacy and the plasmid encoded (DNA) vaccines gave hope for improved protection, as seen for infectious hematopoietic necrosis virus (IHN) (Corbeil et al., 2000; Garver et al., 2005; Traxler et al., 1999). The SAV-3 plasmid vaccine encoding the structural polyprotein C-E3-E1-6K-E2 gives reduced virus load post experimental challenge (numerically)

compared to IWV but not statistically significant (Thorarinsson et al., 2021)). Plasmid vaccines are presumed to attract more B-cells, T-cells, and antigen-presenting cells to the vaccine injection site (Sobhkhez et al., 2018). Despite good, marked penetration, the PD vaccines available do not confer an optimal immunity and protection against disease.

Importance of antibodies in protection against PD

Protection following passive immunization has been documented for PD. The findings confirmed the 100% neutralization after 4, 8, and 15 weeks with antisera raised in salmon by injecting the infective kidney homogenate and after 8 and 15 weeks with cohabitation. Furthermore, results showed significant neutralization with 1:1000 dilution of antisera (Houghton & Ellis, 1996).

Since, 1930 neutralizing antibodies have been studied to uncover the way of action to inactivate virus vaccines. A considerable number of antibodies are necessary to prevent infection. The affinity of neutralizing antibodies can be measured and compared with vaccine efficacy. The discovery of intracellular factor TRIM-21 which determines the extent of neutralization in adenoviruses has opened a new area of understanding neutralization, especially in naked viruses. Finally, the mechanism of virus neutralization, its measurements, kinetics, and efficacy can be addressed in a more accurate way (Klasse, 2014). Hence, to understand the factors influencing the outcome of vaccination, the immune responses generated should be studied in detail and in this study the aim was to characterize the humoral immune response following vaccination of Atlantic salmon with inactivated and plasmid encoded (DNA) vaccines. A combined delivery of simultaneous injection with DNA (im) and an IWV vaccine (ip) was also included in this study.

Estimation of virus neutralization through an indirect fluorescent antibody test (IFAT)

A modified VN test has been used to characterize the immune response to SAV-3. The conventional virus neutralization (VN) assay is based on the presence or absence of cytopathogenic effect (CPE) carried out on fish in the cell line in aquaculture. This assay was modified using an anti-SAV monoclonal antibody to detect virus infected cells through an immune-peroxidase-based immunostaining technique. The test normally takes three (3) days to complete. Testing 352 sera from farmed Atlantic salmon and 302 samples from trout with both assays, 97.72%, and 96.03% correlation was shown (Graham et al., 2003).

OBJECTIVES OF THE STUDY

The overall objective of the study was to characterize the humoral immune response elicited following vaccination with whole virus inactivated and plasmid-encoded (DNA) vaccines in Atlantic salmon.

SUB OBJECTIVES

- Quantification of antibody levels in plasma samples after vaccination of Atlantic salmon with inactivated whole virus and plasmid vaccines using an indirect enzyme-linked immunosorbent assay (ELISA)
- Study the neutralizing activity in plasma samples after immunization with inactivated whole virus and plasmid vaccines through using a neutralization test using CHSE-214 and CHH-1 cell lines.
- Study the impact of neutralizing activity of the same plasma samples after the addition of complement
- Test a neutralization test based on immunofluorescent antibody test (IFAT)

MATERIALS AND METHODS

Vaccine groups and plasma samples

Plasma samples were collected from Atlantic salmon (*Salmo salar* L.) vaccinated under field conditions with a commercial, multivalent inactivated whole virus vaccine (IWV, 0.1ml/fish) given by the intraperitoneal (ip) route, termed mu-IWV, or a plasmid vaccine (DNA, 0.05ml/fish) vaccine intramuscularly (im), termed DNA. Blood samples were collected in heparin tubes (5 ml) followed by centrifugation at 3500 rpm. Plasma was collected by aspiration and transferred to 2 ml tubes and frozen at – 20 °C. Samples were collected >700 degree days post vaccination.

A second group included Atlantic salmon vaccinated with a monovalent commercial inactivated PD vaccine intraperitoneally (ip, 0.05 ml/fish), termed mo-IWV, and a separate group given the monovalent PD vaccine ip and at the same time these fish were injected im with a commercial plasmid vaccine (DNA, 0.05 ml/fish), a combined modality, termed comb-mod.

Plasma samples were analyzed by an ELISA method and for neutralizing activity, with or without the addition of complement from fresh plasma samples (see below), and an immuno-fluorescent neutralizing antibody test (IFAT). Chinook salmon embryo-214 (CHSE-214) Chum salmon Heart-1 (CHH-1) cell lines were used during the study. The tissue culture infective $dose_{50}$ ($TCID_{50}$) was calculated by titration using the CHH-1 cell line.

CELL CULTURE

CHSE-214 and CHH-1 cell lines were originally obtained from the European Collection of Authenticated Cell Cultures (ECACC) were maintained using L-15-Glutamax media (Thermo-Fisher) supplemented with 5 % FBS and kept at 20 ˚C. The cells were incubated at 15 ˚C during virus infection and propagation.

PREPARATION OF CHINOOK SALMON EMBRYO-214 (CHSE-214) CELL LINE AND CHUM SALMON HEART (CHH-1) CONFLUENT CELL MONOLAYERS

Two 25 cm^2 cell culture flasks containing the 90-100 % confluent monolayer of CHSE-214 and CHH-1 cells were washed two times with phosphate-buffered saline (PBS; pH:7.2 ± 0.2) and then incubated with Trypsin-EDTA solution (Sigma Aldrich) to dissociate the cells. L-15 media containing 10% FBS was then added to stop trypsinization and the cells were seeded into 96-well

plates in a 1:4 ratio (approximately 2500 cells/well). The plates were then incubated at 20 ˚C for 72 hours to obtain monolayers with 75 % confluency.

SOURCE OF SALMONID ALPHAVIRUS TYPE 3 (SAV-3)

The SAV-3 isolate was obtained as described (Xu et al., 2010). The virus stock has been stored at -80 ºC and the initial virus titer was 1×10^8 $TCID_{50}$/ml. This stock virus was used to infect CHH-1 cells in order to produce a fresh virus.

VIRUS PROPAGATION ON CHUM SALMON HEART-1 (CHH-1) CELL LINE

About 80% of confluent CHH-1 cells seeded in a 75 cm^2 flask (Corning®) were used for virus propagation. At the time of infections, media was replaced with L_{15}-Glutamax media containing 5% FBS and 50µg/ ml gentamycin (Sigma Aldrich). The flasks were then infected with 200µl of the SAV-3 H10 isolate and incubated at 15 ˚C for ten days (Xu et al., 2010). The occurrence of full cytopathogenic effect (CPE) was observed after seven days and re-observed after ten days. After confirmation of cytopathogenic effect (CPE), the infected monolayer in the flask was frozen and thawed at -20 ˚C and 4 ˚C. Furthermore, the supernatant containing the virus was centrifuged at 25,000 revolutions per minute (rpm) for 10 minutes at 4 ˚C. Finally, a supernatant of 12.5 ml was transferred to 15 ml sterile plastic tubes (12.5ml×2 tubes) and one was stored at 4 ˚C for tissue culture infective $dose_{50}$ ($TCID_{50}$) calculation, and the other one was divided into two parts and stored at -20 ˚C and -80 ˚C for further use during the study.

CALCULATION OF SAV-3 TISSUE CULTURE INFECTIVE $DOSE_{50}$ ($TCID_{50}$)

The tissue culture infective $dose_{50}$ ($TCID_{50}$) was calculated according to Reed and Munch method (Lei et al., 2021). To calculate $TCID_{50}$, a confluent monolayer of chum salmon heart (CHH-1) cell line and reference SAV-3 were selected. A series of 10-fold dilutions ranging from 10^{-1} to 10^{-8} were prepared and then used to inoculate 96 wells plates (Sarsted) containing 75 % confluent monolayers of CHH-1 cells, six well replicates for each dilution. After seven (7) days of incubation at 15˚C, the wells of the plates were observed using a Nikon inverted microscope (Model-ECLIPSE Ts2, 138972**)** and CPE was recorded. The reading was confirmed after 10 days of incubation and the virus titer was estimated using the Spearman & Kärber method (Kärber, 1931).

EFFECT OF PRESERVATION ON TISSUE CULTURE INFECTIVE DOSE$_{50}$ (TCID$_{50}$) OF SAV-3

The SAV-3 was stored at 4 ˚C, -20 ˚C, and -80 ˚C and evaluated for the effect of preservation on TCID$_{50}$. The virus preserved in each temperature was 10-fold diluted from 10^{-1}-10^{-8} and the different dilutions were inoculated into 96 wells plates containing 75 % confluent CHH-1 cells as described above. Positive and negative controls were included.

INDIRECT ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The virus was coated at 100 TCID$_{50}$ of SAV-3, diluted in coating buffer (carbonate: bicarbonate buffer. pH:7.2 ± 0.2). 100 µl of virus suspension was added to each well of the polystyrene plastic 96-well ELISA plate and the plates were incubated at 4 ˚C for overnight. The next day, the plates were washed with washing buffer (phosphate-buffered saline containing 0.5ml/L Tween-20 (PBST-20) three times using 250 µl of volume in each well. The plates were then blocked with 250 µl/well of blocking buffer consisting of 5 % skimmed milk powder in PBST-20. The plates were incubated at room temperature for two hours followed by three times washing using 250 µl of washing buffer per well. Different dilutions, 1:50, 1:100, 1:200, 1:400, 1:800 of each field plasma samples, were prepared in diluent buffer (1% skimmed milk powder in PBST-20) and then added in duplicate wells for each sample. The plates were incubated at room temperature for one hour and then washed five times by adding 250µl of washing buffer per well. Subsequently, 70µl of mouse anti-salmonid (0.5mg/ml) horse radish peroxidase (HRP) labeled antibody (Immunoprecise), diluted 1:1000 in the diluent buffer, was then added to each well and the plates were incubated for one hour at room temperature. This was followed by washing the plate five times and the addition of 70µl per well substrate solution (TMB). The plates were then incubated for fifteen minutes at room temperature in the dark to allow color development. Finally, 70µl of stop solution (1M HCl) was added to each well of the 96-well plate to stop the reaction. The optical density (OD) was then read at 450 nm using Spark multimode microplate reader (Tecan).

VIRUS NEUTRALIZATION (VN) TEST

Virus neutralization (VN) test was performed as described (Rowley et al., 1998) with some modifications. Fifteen 96 well plates each of CHSE-214 and CHH-1 cells with 75 % confluency were used. Fourteen plates of each cell line were used for the neutralization assay while one plate acted as a normal cell control. The field plasma samples were diluted at 1:20, 1:40, 1:80, and 1:160 and incubated with 100 TCID$_{50}$ of SAV-3 for one hour at room temperature. The serum/virus

mixture was then added to the 96 well plates seeded with CHSE-214 and CHH-1 cell lines after removing the old media. The plates were incubated at 15 ˚C for ten days. The results were recorded by observing the neutralizing effect (absence of CPE) using a microscope (Nikon; Model-ECLIPSE Ts2, 138972) after seven and ten days of incubation. The Invitrogen EVOS^{TM}-M5000 imaging (NMBU-ID 18816, LN2-U-094, Ref-AMF5000, SN-F1720-225A-0320) system by Thermo-fisher scientific, Bothell, WA, USA, were used to take pictures of each well on the whole plate along with controls.

EFFECT OF COMPLEMENT ON VIRUS NEUTRALIZATION (VN) TEST USING THE CHINOOK SALMON EMBRYO-214 (CHSE-214) AND CHUM SALMON HEART (CHH-1) CELL LINE

To test the effect of complement on virus neutralization, the same protocol as described above in neutralization assay was repeated for both cell lines (CHSE-214, CHH-1) adding 3µl of fresh salmon plasma to 32µl each of the serum samples before incubation with the virus for 1 hour.

IMMUNOFLUORESCENT ANTIBODY TEST (IFAT) FOR ASSESSMENT OF VIRUS NEUTRALIZATION

The neutralization test was performed as described (Gao et al., 2021) with some modifications. Six plasma samples from both groups were diluted in maintenance culture media containing 1% fetal bovine serum (FBS) and incubated for one (1) hour with 100 particles of SAV-3 before being seeded into 96-well plates containing CHH-1 cell line. Six replicates were used for each sample. After 72-96 hours of incubation at 15 ˚C, cells were fixed with a 1:1 ratio of acetone and methanol and subsequently assayed with IFAT. Anti-E2 polyclonal antibody (17H23) and Alexa-fluor 488 labeled anti-mouse IgG (Thermo-Fischer) were used to detect the virus-positive cells. After IFAT stanning, the stained cells were visualized using the fluorescence microscope (Olympus- Model-U-LH100HG, No. 3D09579, Olympus Optical Co., Ltd., Made in Japan), and both virus-positive and negative cells were counted.

IFAT PROCEDURE

The six field plasma samples from both groups were diluted at 1:20, 1:40, and 1:80 in a cell culture maintenance medium containing 1% FBS. 100 $TCID_{50}$ of the SAV-3 was used from 1.5×10^{7} titrated SAV-3 virus. The 50µl of plasma sample and 50µl of 100 $TCID_{50}$ SAV-3 were mixed and incubated at room temperature for 1 hour. Six replicates were used for each dilution of each sample. After 1h, the plasma-virus mixture was added to the 75 % confluent monolayer of the CHH-1 cell line in

96-well plates. After two hours of adsorption, the whole layer was washed two times with PBS, pH 7.2. Finally, 100µl of maintenance media was added in all wells of the plate and incubated at 15 ^{o}C for 72 hours (3 days) along with controls.

On the second day, the plates were agitated between all washing and antibody incubation steps. The maintenance cell culture media from a 96-well plate containing an infected CHH-1 cell line was discarded. The infected cells were fixed by adding 70µl of fixative containing methanol: acetone (1:1) for 20 minutes at 4 °C. Cells were washed twice for five minutes each time using 100µl of phosphate-buffered saline (PBS), pH 7.2. Blocking was done by adding 100µl of 5% dry skimmed milk powder solution in PBS (2.5 g of dry milk in 50 ml of PBS) for 2h at room temperature. Then 70µl of primary antibody was added, rabbit anti-E2 (Xu et al., 2012), and diluted 1:500 in 5 % dry skimmed milk (16µl in 8 ml of 5 % dry skimmed milk). Plates were then incubated for 1h at room temperature, followed by 3x washing, five minutes each with 100µl of PBS, pH7.2. Then 70µl of fluorescence-labeled secondary antibody, Alexa fluor 488 (Life Technologies) anti-rabbit IgG diluted 1:500 in 5% skimmed milk (16µl of Alexa in 8ml of 5% skimmed milk) and incubated for 1h at room temperature. Then 70µl of Hoechst staining in PBS (2 µg/ml) and incubated for five minutes at room temperature in the dark. Cells were then washed twice, five minutes each with 100µl of PBS, pH 7.2. Fluid was discarded and replaced with 100µl of fresh PBS, pH 7.2 per well. This was followed by examination in a fluorescence microscope, and cells were counted (n=200) followed by calculation of percent infected cells. Controls were non-infected cell cultures and positive control were included.

Statistical methods

The obtained OD and other data material were organized in Excel and imported to Stata17 where appropriate for statistical analysis. Antibody levels obtained from ELISA runs were compared between groups using a Kruskal Wallis test after testing for normality of distribution. Statistical differences were considered significant at $p<0.05$.

RESULTS

Antibody levels measured by ELISA are shown in Figure 9 for IWV and DNA vaccinated fish. Circulating antibody levels are higher in DNA compared to IWV vaccinated fish (numerically) but there was no significant difference between these two groups at either dilution ($p>0.05$). For both vaccines, OD values were significantly higher than controls ($p=0.001$, Kruskal–Wallis equality-of-populations rank test).

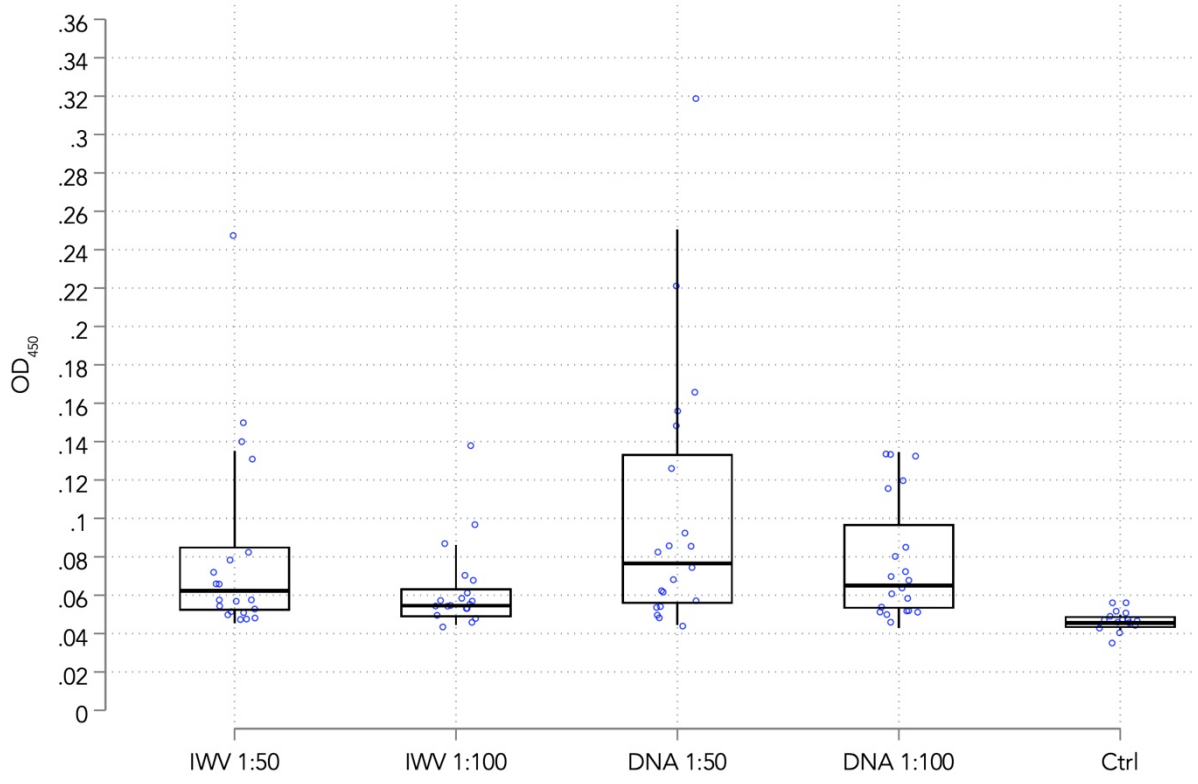


Figure 9. Antibody levels for fish vaccinated with inactivated whole virus (IWV) or DNA vaccines, at different dilution of primary plasma samples. Ctrl is non-vaccinated control fish.

NEUTRALIZATION STUDIES

The initial studies included testing of neutralization using different $TCID_{50}$ of virus incubating with plasma samples diluted 1:40 and 1:80. Three fish from each group vaccinated with DNA, monovalent and multivalent IWV vaccines, and groups vaccinated intranasally and ip (monovalent IWV), respectively, were included in this initial study. The results are summarized in Table 1.

Table 1. Numbers indicate samples that gave neutralization when tested at 1:40 and 1:80 dilution and incubated on CHH-1 cells (out of three fish) for the different vaccine groups at different $TCID_{50}$ of virus.

Dilution	DNA-100	DNA-75	DNA-50	DNA-25	mo-IWV-100	mo-IWV-75	mo-IWV-50	mo-IWV-25	mu-IWV-100	mu-IWV-75	mu-IWV-50	mu-IWV-25
1:40	1/3	1/3	1/3	2/3	0/3	1/3	1/3	1/3	2/3	2/3	2/3	2/3
1:80	1/3	2/3	2/3	2/3	1/3	1/3	1/3	1/3	0/3	0/3	0/3	0/3

Dilution	co-IWV-100	co-IWV-75	co-IWV-50	co-IWV-25
1:40	0/3	0/3	0/3	0/3
1:80	0/3	0/3	0/3	0/3

The results showed that neutralization was obtained at 25 $TCID_{50}$ in 2/3 samples for DNA and mu-IWV at 1:40 dilution, while for mo-IWV only 1/3 neutralized the virus. The combined vaccination modality did not elicit neutralizing antibodies in the fish examined.

NEUTRALIZATION TEST FOR DNA AND mu-IWV VACCINES

As a next step, 20 fish from each of DNA and mu-IWV vaccinated fish were tested for neutralization antibodies, with the plasma dilution 1:20 and twofold to 1:160. The amount of virus used for neutralization was 100 $TCID_{50}$. The main purpose was to document full neutralization (absent of CPE) but since very few samples were found to show any reduction in CPE, a grading of the CPE in cell culture was adopted and scored also as less than 100% CPE when relevant.

The general finding was that no neutralization was observed apart from one fish in the mu-IWV group where a reduction in CPE was seen at 1:40 – 1:160 with 70, 80 and 90 % CPE, respectively. The details of these results are shown in the Appendix.

EFFECT OF COMPLEMENT

As a next step, the impact of adding complement to the plasma samples when performing the neutralization tests. The samples had been collected fresh but frozen and thawed more than one time and thus the likelihood of reducing the amount of complement in the samples was considered high. For this reason, fresh plasma from normal salmon was added to the samples, 3 µl of fresh plasma to 32 µl of plasma collected from vaccinated fish, DNA and mo-IWV vaccines, and then incubated with 100 $TCID_{50}$ virus particles following the same scheme as above. Testing was done on both CHSE-214 and CHH-1 cells.

The plasma samples were diluted as 1:20, 1:40, 1:80, and 1:160 in maintenance media, mixed with 100 $TCID_{50}$ of cell culture-grown SAV-3. Plates were incubated at 15 °C for seven-ten days, adding fresh plasma to study the effect of complement. The results after seven and ten days showed that there was CPE in all sample plates in dilutions 1:40, 1:80, and 1:160. At 1:20 dilution cell toxicity was observed. Adding fresh plasma showed a small reduction in CPE for 4 samples (out of 20 tested) in the IWV group, and in 8/12 in the DNA vaccinated group. The difference was observed as reduced number of cells showing CPE (see Annex for details).

NEUTRALIZATION ASSAYED BY IFAT STAINING

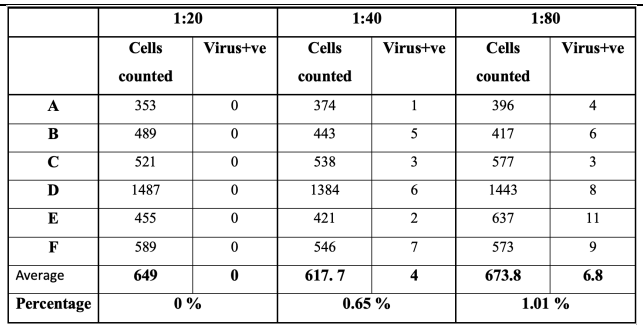
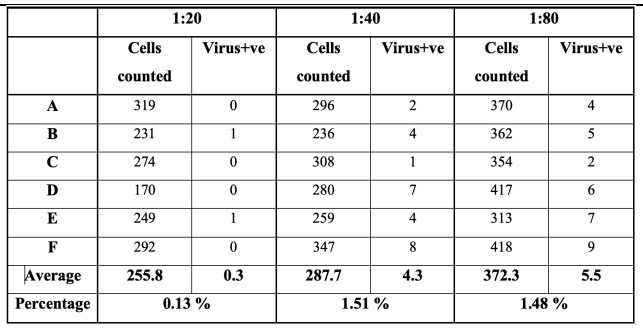
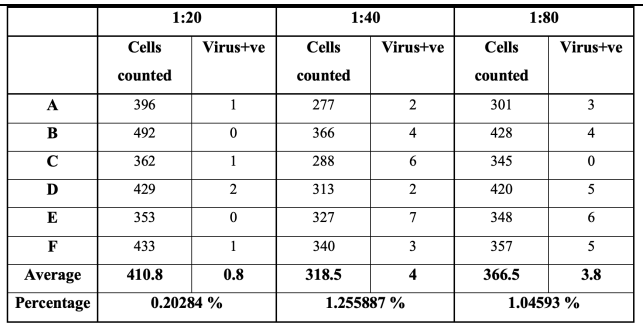
Based on a limited outcome of a classical neutralization test, the next approach was to test the applicability of IFAT staining of infected cell cultures with or without preincubation with plasma from immunized fish. For this purpose, 6 plasma samples from DNA or mu-IWV vaccinated fish were included, and 6 parallel wells were included for each dilution, 1:20 – 1:80 of primary plasma samples.

The number of virus positive cells relative to the number of cells counted, was estimated and compared to the number of virus positive cells in positive controls (without plasma from immunized salmon added). The neutralization titer was given as the dilution where the number of positive cells on average was reduced by 50% and expressed as exact titer or higher than the highest dilution of the primary plasma samples. For several of the plasma samples from the DNA immunized fish, the number of positive cells was far below the 50% reduction of virus positive cells compared to controls, and therefore indicated as much higher than a titer of 80 (>>80). The results are shown in the table below.

Table 2A

	Fish no.					
Vaccines	1	2	3	4	5	6
mu-IWV	40	40	>80	≈80	>80	>80
DNA	40	>>80	>>80	>>80	80	>>80

Table 2B

Vaccine	Results	Estimated titer
Mu-IWV #1		>80
Mu-IWV #2		≈40
Mu-IWV #3		>80

	1:20		1:40		1:80	
	Cells counted	Virus+ve	Cells counted	Virus+ve	Cells counted	Virus+ve
A	353	0	374	1	396	4
B	489	0	443	5	417	6
C	521	0	538	3	577	3
D	1487	0	1384	6	1443	8
E	455	0	421	2	637	11
F	589	0	546	7	573	9
Average	**649**	**0**	**617. 7**	**4**	**673.8**	**6.8**
Percentage	**0 %**		**0.65 %**		**1.01 %**	

	1:20		1:40		1:80	
	Cells counted	Virus+ve	Cells counted	Virus+ve	Cells counted	Virus+ve
A	319	0	296	2	370	4
B	231	1	236	4	362	5
C	274	0	308	1	354	2
D	170	0	280	7	417	6
E	249	1	259	4	313	7
F	292	0	347	8	418	9
Average	**255.8**	**0.3**	**287.7**	**4.3**	**372.3**	**5.5**
Percentage	**0.13 %**		**1.51 %**		**1.48 %**	

	1:20		1:40		1:80	
	Cells counted	Virus+ve	Cells counted	Virus+ve	Cells counted	Virus+ve
A	396	1	277	2	301	3
B	492	0	366	4	428	4
C	362	1	288	6	345	0
D	429	2	313	2	420	5
E	353	0	327	7	348	6
F	433	1	340	3	357	5
Average	**410.8**	**0.8**	**318.5**	**4**	**366.5**	**3.8**
Percentage	**0.20284 %**		**1.255887 %**		**1.04593 %**	

Mu-IWV #4

	Cells counted	Virus+ve	Cells counted	Virus+ve	Cells counted	Virus+ve
A	318	0	372	1	405	2
B	420	0	342	0	351	3
C	357	0	301	2	374	1
D	330	0	362	1	410	6
E	272	0	285	3	449	5
F	381	0	316	2	426	8
Average	**346.3**	**0**	**329.7**	**1.5**	**402.5**	**4.2**
Percentage	**0 %**		**0.46%**		**1.04%**	

>80

Mu-IWV #5

	1:20		1:40		1:80	
	Cells counted	Virus+ve	Cells counted	Virus+ve	Cells counted	Virus+ve
A	365	0	366	2	415	3
B	361	1	387	1	359	2
C	373	0	349	3	388	5
D	370	0	324	1	355	4
E	428	2	413	4	352	6
F	352	0	346	2	281	4
Average	**374.8**	**0.5**	**364.2**	**2.17**	**358.3**	**4**
Percentage	**0.13 %**		**0.59%**		**1.12%**	

80

Mu-IWV #6

	1:20		1:40		1:80	
	Cells counted	Virus+ve	Cells counted	Virus+ve	Cells counted	Virus+ve
A	199	0	305	0	684	0
B	582	0	242	0	819	1
C	280	0	349	1	413	0
D	340	0	920	0	645	0
E	370	0	273	0	511	0
F	636	0	860	0	572	13
Average	**401.2**	**0**	**491.5**	**0.17**	**607.3**	**2.3**
Percentage	**0 %**		**0.034 %**		**0.38 %**	

>>80

Control

	Culture media with 1% FBS cells as a Control		Normal Salmon Plasma + Virus as a Control		Virus as a Control	
	Cells counted	Virus+ve	Cells counted	Virus+ve	Cells counted	Virus+ve
1	449	0	283	6	306	0
2	261	0	243	4	284	6
3	356	0	328	8	268	21
4	378	0	345	8	323	14
5	348	0	345	8	373	10
6	329	0	296	9	360	16
7	358	0	356	3	457	5
8	331	0	351	5	466	4
Average	**351.3**	**0**	**318.4**	**6.4**	**354.6**	**9.5**
Percentage	**0 %**		**2.00 %**		**2.68 %**	

Vaccine	Results	Estimated titer
DNA #1		80
DNA #2		>>80
DNA #3		>>80
DNA #4		>>80

	1:20		1:40		1:80	
	Cells counted	Virus +ve	Cells counted	Virus +ve	Cells counted	Virus +ve
A	680	0	400	3	466	8
B	508	0	500	6	517	17
C	732	1	757	2	449	18
D	727	0	633	15	777	23
E	494	0	447	5	786	6
F	868	1	755	4	573	11
Average	**668.2**	**0.3**	**582**	**5.8**	**594.7**	**13.8**
Percentage	**0.05 %**		**1.0 %**		**2.3%**	

	1:20		1:40		1:80	
	Cells counted	Virus +ve	Cells counted	Virus +ve	Cells counted	Virus +ve
A	393	0	293	2	341	4
B	381	1	331	5	328	7
C	348	0	443	4	357	9
D	284	2	309	3	284	6
E	297	0	268	6	324	4
F	284	1	326	7	341	11
Average	**331.2**	**0.7**	**328.3**	**4.5**	**329.2**	**6.8**
Percentage	**0.2 %**		**1.4 %**		**2.1%**	

	1:20		1:40		1:80	
	Cells counted	Virus +ve	Cells counted	Virus +ve	Cells counted	Virus +ve
A	239	0	342	1	433	3
B	400	0	510	5	576	6
C	376	0	409	1	487	4
D	381	0	192	0	304	1
E	422	0	432	4	489	5
F	402	0	367	2	398	7
Average	**370**	**0**	**375.3**	**2.2**	**447.8**	**4.3**
Percentage	**0 %**		**0.6 %**		**1.0 %**	

	1:20		1:40		1:80	
	Cells counted	Virus+ve	Cells counted	Virus+ve	Cells counted	Virus+ve
A	309	1	383	2	361	5
B	444	0	377	1	425	6
C	386	0	388	2	490	8
D	269	1	345	0	315	12
E	437	1	438	5	507	3
F	379	0	362	1	403	0
Average	**370.7**	**0.5**	**382.2**	**1.8**	**416.8**	**5.7**
Percentage	**0.1%**		**0.5 %**		**1.4 %**	

DNA #5

	1:20		1:40		1:80	
	Cells counted	Virus+ve	Cells counted	Virus+ve	Cells counted	Virus+ve
A	438	0	406	4	322	8
B	193	0	446	2	353	12
C	408	1	464	5	417	9
D	413	0	378	3	350	13
E	401	0	325	7	259	8
F	342	0	361	5	401	6
Average	**365.8**	**0.2**	**396.7**	**4.3**	**350.3**	**9.3**
Percentage	**0.1**		**1.1 %**		**2.7 %**	

>80

DNA #6

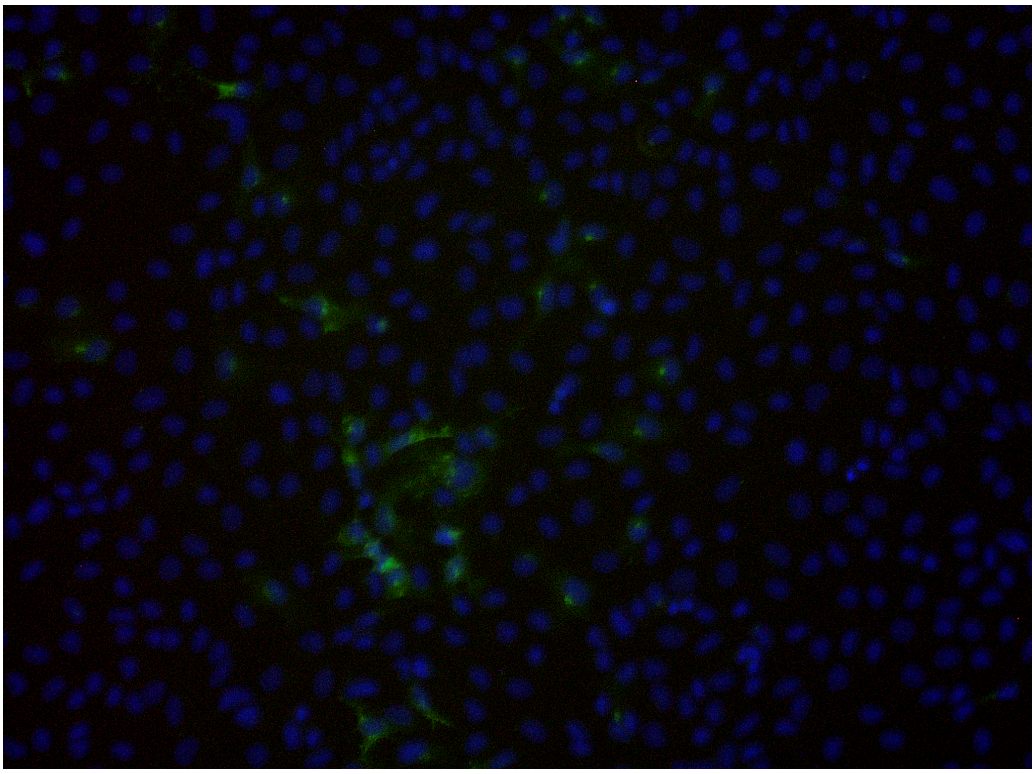
	1:20		1:40		1:80	
	Cells counted	Virus+ve	Cells counted	Virus+ve	Cells counted	Virus+ve
A	1110	0	1040	0	995	0
B	716	0	1238	3	1007	4
C	785	2	914	0	1002	0
D	930	0	811	1	943	3
E	1158	0	844	0	988	0
F	1526	3	998	5	1214	4
Average	**1037.5**	**0.8**	**974.2**	**1.5**	**1024.8**	**1.8**
Percentage	**0.1 %**		**0.2 %**		**0.2 %**	

>>>80

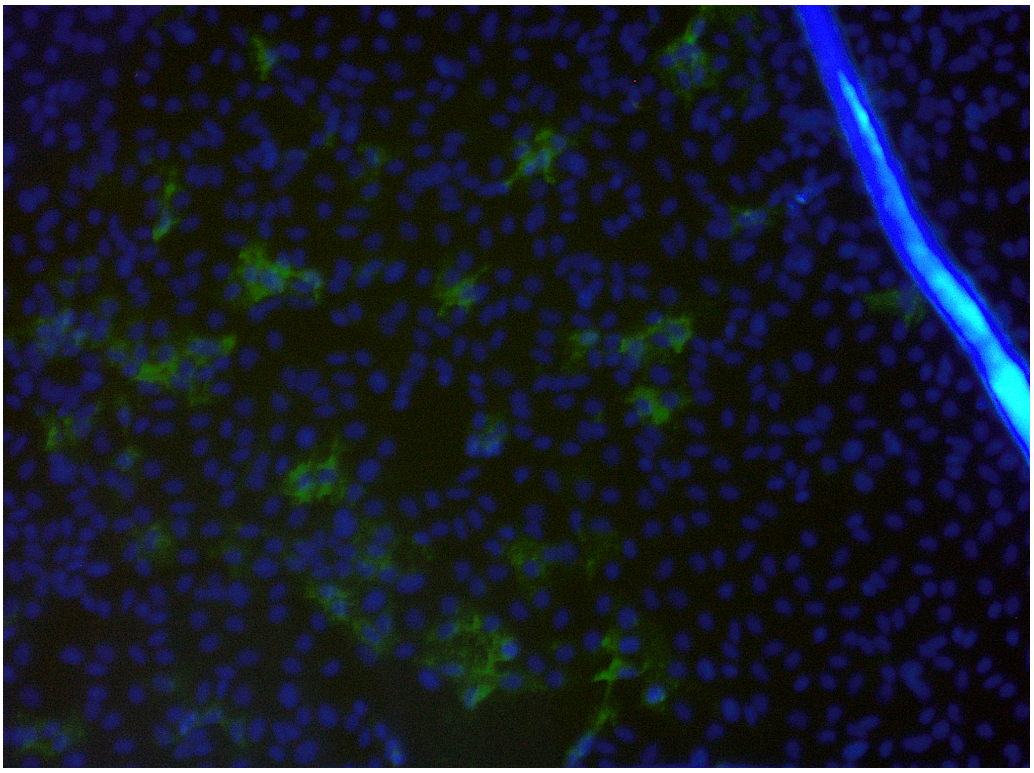
Control

	Culture media with 1% FBS cells as a Control		Normal Salmon Plasma + Virus as a Control		Virus as a Control	
	Cells counted	Virus+ve	Cells counted	Virus+ve	Cells counted	Virus+ve
1	776	0	684	58	536	42
2	950	0	614	64	622	51
3	798	0	702	61	682	49
4	448	0	335	14	311	6
5	413	0	355	11	360	4
6	320	0	432	12	363	24
7	477	0	451	7	422	6
8	453	0	465	5	433	4
Average	**579.4**	**0**	**504.8**	**29**	**466.1**	**23.3**
Percentage	**0 %**		**5.7 %**		**5.0 %**	

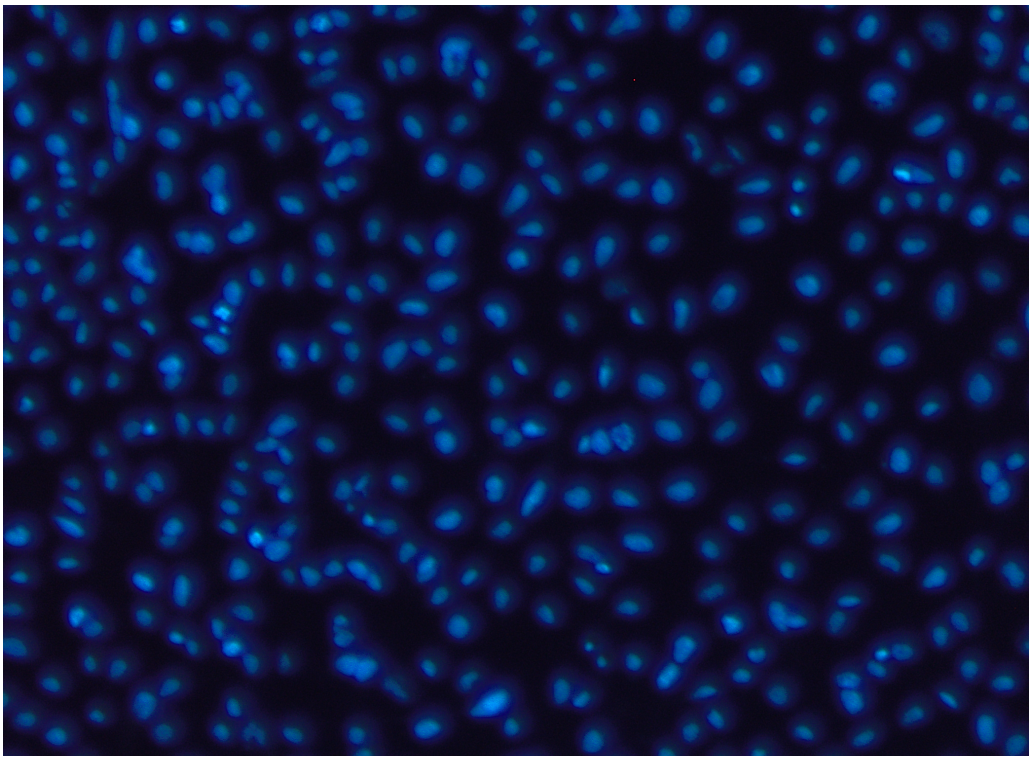
Examples of IFAT staining are shown in Figure 10.



Sample added plasma from DNA vaccinated fish



Virus positive control (no plasma added)



Cell control

Figure 10. IFAT staining results with our without pre-incubation with immune plasma samples

DISCUSSION

This study started by analyzing the level of antibodies in samples based on an enzyme-linked immunosorbent assay (ELISA), and in general the level of circulating antibodies was low in all groups although significantly increased compared to non-vaccinated controls. Next a standard VNT was carried out and the general finding was that variable results were obtained using this approach, and it was not possible to draw a firm conclusion as regards level of neutralizing antibodies in vaccinated groups, irrespective of vaccines used.

On this basis we used a different approach to test for neutralizing antibodies based on infecting permissive cell cultures after mixing immune plasma and virus prior to addition onto the cell cultures, and then detecting presence of infection and replication of virus through coloration with a virus-specific antibody. This proved to yield better, and more consistent results and a certain level of neutralizing titer was detected in vaccinated fish. While this method still needs to be validated in the laboratory it has been used by several laboratories for assessment of antibodies against PD virus (Thorarinsson et al., 2021). The method is somewhat time consuming as there is need for including a relatively higher number of cells when counting virus positive and negative cells but this can be done by obtaining photographs of cell cultures and potentially in the future, it could also be subject to automatic counting using self-learning programs. The read-out is an estimation of reduced level of virus positive cells in the parallels incubated with immune serum from vaccinated salmon compared to infected controls (without serum added). We included 6 parallel wells for this purpose. The dilution series should be optimized since only three dilutions (1:20-1:80, 2 fold) were included in this study, and to get more precise estimate of the final titer additional parallels should be included. The dilution series included was based on results from classical VNT

studies and limitation of time did not allow for additional testing. This is obviously a topic for future testing and development.

The results obtained using the IFAT neutralization test align with previous studies, where the same method was used (Thorarinsson et al., 2021), and the same relationship between IWV and DNA vaccines was reported. The results are in agreement with a previous study (Cao et al., 2017) but they used monoclonal antibodies reactive with 3 subtypes of the virus. Further, plasmid-encoded (DNA) vaccines have been shown to produce significantly higher anti-SAV3 neutralizing antibody titers, lower viremia, and reduced transmission to the naive fish. It not only protects the fish against the destruction of the pancreas and reduced growth but also increased post-challenge survival. The results are not in line with an earlier study (Xu et al., 2012) where it was shown that a IWV vaccine is superior in immunogenicity over sub-unit and plasmid-encoded (DNA) vaccines. They compared the immunogenicity and protection against mortality for whole virus-inactivated and spike protein sub-unit and plasmid-encoded (DNA) vaccines in their investigation. They used the water in oil emulsion of sub-unit and inactivated and non-formulated plasmid-encoded (DNA) vaccines. During the challenge, they found circulating neutralizing antibodies with marginal protection, a decrease in virus and mortality for the whole virus inactivated, and E_2 subunit vaccine. There were 3 logs (10) reduction for the virus, protection of internal organs for pathology in the whole virus-inactivated vaccine. The E1 sub-unit vaccine also showed significant protection but not neutralizing antibodies. The E1 and E2 plasmid-encoded (DNA) vaccines showed marginal protection and low reduction of viral replication in target organs. Moreover, challenge showed an upregulation of IFN-γ and IL2-mRNA expression, which was interpreted as a potential explanation for the protection seen despite antibody levels being low.

Previous studies have advocated virus neutralization test for the diagnosis and in epidemiological studies of pancreas disease in Atlantic salmon (Graham et al., 2005). Further to this it has been stated that a low number of antibodies are required to neutralize free virus infections and a high amount is required to control cell-to-cell spread (Parren & Burton, 2001). He also stated that detectable anti-SAV3 antibodies are present in plasma of vaccinated salmon but also found a lack of complete neutralization using CHH-1 and CHSE-214 cell lines. This again contradicts previous findings that reported 100 % neutralization and showed that antisera raised by intraperitoneal injection of infective kidney homogenate or experimental infection and cohabitation with infected fish induce high levels of neutralizing antibodies (Houghton & Ellis, 1996) was based on natural infection and sampling was carried out from survivors, meaning that in principle a live vaccination regime was used. While obviously not directly comparable to standard vaccination studies, the seminal study carried out by Houghton and Ellis (Houghton & Ellis, 1996), documents the potential of the strength of the immune response given that an appropriate or optimal vaccination regime is used.

Finally, after analyzing the results vaccines samples, plasma samples of both groups through neutralization, role of complement in neutralization, quantification of antibodies through ELISA and estimation of virus infected cells through IFAT, it is recommended to combine the ELISA and the IFAT neutralization test for detection and characterization of antibodies. Earlier studies have found low agreement between IgM assays (ELISA) and IFAT neutralization tests in studies conducted by (Gao et al., 2019). They recommended combination of ELISA and IFAT VNT for detection of antibodies post vaccination.

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Appendix

(Part-1) 4-Vaccines

Plasmid-encoded vaccine (DNA) (1:40)

	Sample 1				Sample 2				Sample 3			
	1	2	3	4	5	6	7	8	9	10	11	12
	100	75	50	25	100	75	50	25	100	75	50	25
1:40A	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Negative	Negative	Negative	Negative
1:40B	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Negative	Negative	Negative	Negative
1:40C	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Negative	Negative	Negative	Negative
1:40D	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Negative	Negative	Negative	Negative
1:40E	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Negative	Negative	Negative	Negative
1:40F	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Negative	Negative	Negative	Negative
G-Controls	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus
H-Controls	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus

Plasmid-encoded vaccine (DNA) (1:80)

	Sample 1				Sample 2				Sample 3			
	1	2	3	4	5	6	7	8	9	10	11	12
	100	75	50	25	100	75	50	25	100	75	50	25
1:80A	Positive	Positive	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
1:80B	Positive	Positive	Positive	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
1:80C	Positive	Positive	Positive	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
1:80D	Positive	Positive	Positive	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
1:80E	Positive	Positive	Positive	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
1:80F	Positive	Positive	Positive	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
G-Controls	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus

H-Controls	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus

Alpha-ject (1:40)

	Sample 1				Sample 2				Sample 3			
	1	2	3	4	5	6	7	8	9	10	11	12
	100	75	50	25	100	75	50	25	100	75	50	25
1:40A	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Negative	Negative
1:40B	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Negative	Negative
1:40C	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Negative	Negative
1:40D	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Negative	Negative
1:40E	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Negative	Negative
1:40F	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Negative	Negative
G-Controls	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus
H-Controls	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus

Alpha-ject (1:80)

	Sample 1				Sample 2				Sample 3			
	1	2	3	4	5	6	7	8	9	10	11	12
	100	75	50	25	100	75	50	25	100	75	50	25
1:80A	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Negative	Negative	Negative
1:80B	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Negative	Negative	Negative
1:80C	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Negative	Negative	Negative
1:80D	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Negative	Negative	Negative
1:80E	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Negative	Negative	Negative
1:80F	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Negative	Negative	Negative
G-Controls	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus

H-Controls	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus

Kombi (1:40)

	Sample 1				Sample 2				Sample 3			
	1	2	3	4	5	6	7	8	9	10	11	12
	100	75	50	25	100	75	50	25	100	75	50	25
1:40A	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
1:40B	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
1:40C	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
1:40D	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
1:40E	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
1:40F	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
G-Controls	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus
H-Controls	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus

Kombi (1:80)

	Sample 1				Sample 2				Sample 3			
	1	2	3	4	5	6	7	8	9	10	11	12
	100	75	50	25	100	75	50	25	100	75	50	25
1:80A	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
1:80B	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
1:80C	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
1:80D	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
1:80E	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
1:80F	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
G-Controls	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus

H-Controls	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus

MSD Vaccine (1:40)

	Sample 5				Sample 6				Sample 8			
	1	2	3	4	5	6	7	8	9	10	11	12
	100	75	50	25	100	75	50	25	100	75	50	25
1:40A	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Positive	Positive	Positive
1:40B	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Positive	Positive	Positive
1:40C	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Positive	Positive	Positive
1:40D	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Positive	Positive	Positive
1:40E	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Positive	Positive	Positive
1:40F	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Positive	Positive	Positive
G-Controls	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus
H-Controls	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus

MSD Vaccine (1:80)

	Sample 5				Sample 6				Sample 8			
	1	2	3	4	5	6	7	8	9	10	11	12
	100	75	50	25	100	75	50	25	100	75	50	25
1:80A	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
1:80B	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
1:80C	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
1:80D	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
1:80E	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
1:80F	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
G-Controls	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus

H-Controls	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus

(Part-2) Neutralization on CHSE-214 & CHH-1 cell lines

Same results on both the cell lines

M	Sample 1				Sample 2				Sample 3			
	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160
	1	2	3	4	5	6	7	8	9	10	11	12
A	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
B	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
C	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
D	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
E	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
F	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
G	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus
H	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus

M	Sample 4				Sample 5				Sample 6			
	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160
	1	2	3	4	5	6	7	8	9	10	11	12
A	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
B	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
C	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
D	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
E	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
F	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
G	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus
H	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus

M	Sample 7				Sample 8				Sample 9			
	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160
	1	2	3	4	5	6	7	8	9	10	11	12
A	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
B	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
C	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
D	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
E	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
F	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
G	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus
H	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus

M	Sample 10				Sample 11				Sample 12			
	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160
	1	2	3	4	5	6	7	8	9	10	11	12
A	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
B	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
C	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
D	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
E	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
F	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
G	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus
H	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus

M	Sample 13				Sample 14				Sample 15			
	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160
	1	2	3	4	5	6	7	8	9	10	11	12
A	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
B	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive

C	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
D	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
E	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
F	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
G	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus
H	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus

M	Sample 16				Sample 17				Sample 18			
	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160
	1	2	3	4	5	6	7	8	9	10	11	12
A	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
B	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
C	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
D	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
E	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
F	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
G	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus
H	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus

M	Sample 19				Sample 20				CM Control			
	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160
	1	2	3	4	5	6	7	8	9	10	11	12
A	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Negative	Negative	Negative	Negative
B	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Negative	Negative	Negative	Negative
C	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Negative	Negative	Negative	Negative
D	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Negative	Negative	Negative	Negative
E	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Negative	Negative	Negative	Negative
F	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Negative	Negative	Negative	Negative
G	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus

H	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus

K	Sample 1				Sample 2				Sample 3			
	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160
	1	2	3	4	5	6	7	8	9	10	11	12
A	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
B	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
C	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
D	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
E	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
F	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
G	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus
H	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus

K	Sample 4				Sample 5				Sample 6			
	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160
	1	2	3	4	5	6	7	8	9	10	11	12
A	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
B	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
C	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
D	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
E	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
F	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
G	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus
H	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus

K	Sample 7				Sample 8				Sample 9			
	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160
	1	2	3	4	5	6	7	8	9	10	11	12

A	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
B	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
C	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
D	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
E	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
F	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
G	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus
H	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus

K	Sample 10				Sample 11				Sample 12			
	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160
	1	2	3	4	5	6	7	8	9	10	11	12
A	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
B	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
C	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
D	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
E	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
F	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
G	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus
H	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus

K	Sample 13				Sample 14				Sample 15			
	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160
	1	2	3	4	5	6	7	8	9	10	11	12
A	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
B	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
C	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
D	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
E	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive

F	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
G	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus
H	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus

K	Sample 16				Sample 17				Sample 18			
	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160
	1	2	3	4	5	6	7	8	9	10	11	12
A	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
B	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
C	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
D	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
E	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
F	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
G	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus
H	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus

K	Sample 19				Sample 20				CM Control			
	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160
	1	2	3	4	5	6	7	8	9	10	11	12
A	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Negative	Negative	Negative	Negative
B	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Negative	Negative	Negative	Negative
C	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Negative	Negative	Negative	Negative
D	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Negative	Negative	Negative	Negative
E	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Negative	Negative	Negative	Negative
F	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Negative	Negative	Negative	Negative
G	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus
H	CM	CM	CM	CM	NS	NS	NS	NS	Virus	Virus	Virus	Virus

(Part-3) Effect of complement on Neutralization for CHSE-214 & CHH-1 cell lines

Same results for both the cell lines

M-Samples

M	Sample 1				Sample 2				Sample 3			
	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160
	1	2	3	4	5	6	7	8	9	10	11	12
A	Toxicity	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
B	Toxicity	50% CPE	Positive	Positive	Positive	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
C	Toxicity	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
D	Toxicity	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
E	Toxicity	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
F	Toxicity	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
G	Negative	Negative	Negative	Negative	Blank	Toxicity	Toxicity	Toxicity	Positive	Positive	Positive	Positive
H	Negative	Negative	Negative	Negative	Blank	Toxicity	Toxicity	Toxicity	Positive	Positive	Positive	Positive

M	Sample 4				Sample 5				Sample 6			
	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160
	1	2	3	4	5	6	7	8	9	10	11	12
A	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
B	Toxicity	50% CPE	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
C	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
D	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
E	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
F	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
G	Negative	Negative	Negative	Negative	Toxicity	Toxicity	Toxicity	Toxicity	Positive	Positive	Positive	Positive
H	Negative	Negative	Negative	Negative	Blank	Blank	Toxicity	Toxicity	Positive	Positive	Positive	Positive

M	Sample 7				Sample 8				Sample 9			
	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160

	1	2	3	4	5	6	7	8	9	10	11	12
A	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
B	Toxicity	50% CPE	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
C	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
D	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
E	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
F	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
G	Negative	Negative	Negative	Negative	Toxicity	Toxicity	Toxicity	Toxicity	Positive	Positive	Positive	Positive
H	Negative	Negative	Negative	Negative	Toxicity	Toxicity	Toxicity	Toxicity	Positive	Positive	Positive	Positive

M	Sample 10				Sample 11				Sample 12			
	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160
	1	2	3	4	5	6	7	8	9	10	11	12
A	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
B	Toxicity	50% CPE	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
C	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
D	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
E	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
F	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
G	Negative	Negative	Negative	Negative	Toxicity	Toxicity	Toxicity	Toxicity	Positive	Positive	Positive	Positive
H	Negative	Negative	Negative	Negative	Toxicity	Toxicity	Toxicity	Toxicity	Positive	Positive	Positive	Positive

M	Sample 13				Sample 14				Sample 15			
	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160
	1	2	3	4	5	6	7	8	9	10	11	12
A	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
B	Toxicity	50% CPE	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
C	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
D	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive

E	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
F	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive
G	Negative	Negative	Negative	Negative	Toxicity	Toxicity	Toxicity	Toxicity	Positive	Positive	Positive	Positive
H	Negative	Negative	Negative	Negative	Toxicity	Toxicity	Toxicity	Toxicity	Positive	Positive	Positive	Positive

M	Sample 16				Sample 17				Sample 18			
	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160
	1	2	3	4	5	6	7	8	9	10	11	12
A	Toxicity	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE
B	Toxicity	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE
C	Toxicity	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE
D	Toxicity	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE
E	Toxicity	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE
F	Toxicity	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE	60%CPE
G	Negative	Negative	Negative	Negative	Toxicity	Toxicity	Toxicity	Toxicity	Positive	Positive	Positive	Positive
H	Negative	Negative	Negative	Negative	Toxicity	Toxicity	Toxicity	Toxicity	Positive	Positive	Positive	Positive

M	Sample 19				Sample 20				Cell culture media control			
	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160
	1	2	3	4	5	6	7	8	9	10	11	12
A	Positive	Positive	50%CPE	50%CPE	Toxicity	Positive	Positive	Positive	Negative	Negative	Negative	Negative
B	Positive	Positive	50%CPE	50%CPE	Toxicity	Positive	Positive	Positive	Negative	Negative	Negative	Negative
C	Positive	Positive	50%CPE	50%CPE	Toxicity	Positive	Positive	Positive	Negative	Negative	Negative	Negative
D	Positive	Positive	50%CPE	50%CPE	Toxicity	Positive	Positive	Positive	Negative	Negative	Negative	Negative
E	Positive	Positive	50%CPE	50%CPE	Toxicity	Positive	Positive	Positive	Negative	Negative	Negative	Negative
F	Positive	Positive	50%CPE	50%CPE	Toxicity	Positive	Positive	Positive	Negative	Negative	Negative	Negative
G	Negative	Negative	Negative	Negative	Toxicity	Toxicity	Toxicity	Toxicity	Positive	Positive	Positive	Positive
H	Negative	Negative	Negative	Negative	Toxicity	Toxicity	Toxicity	Toxicity	Positive	Positive	Positive	Positive

K-Samples

K	Sample 1				Sample 2				Sample 3			
	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160
	1	2	3	4	5	6	7	8	9	10	11	12
A	Toxicity	Positive	50%CPE	50%CPE	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	50%CPE
B	Toxicity	Positive	50%CPE	50%CPE	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	50%CPE
C	Toxicity	Positive	50%CPE	50%CPE	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	50%CPE
D	Toxicity	Positive	50%CPE	50%CPE	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	50%CPE
E	Toxicity	Positive	50%CPE	50%CPE	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	50%CPE
F	Toxicity	Positive	50%CPE	50%CPE	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	50%CPE
G	Negative	Negative	Negative	Negative	Blank	Toxicity	Toxicity	Toxicity	Positive	Positive	Positive	Positive
H	Negative	Negative	Negative	Negative	Blank	Toxicity	Toxicity	Toxicity	Positive	Positive	Positive	Positive

K	Sample 4				Sample 5				Sample 6			
	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160
	1	2	3	4	5	6	7	8	9	10	11	12
A	Toxicity	Positive	Positive	60%CPE	Toxicity	Positive	Positive	70%CPE	Toxicity	Positive	Positive	80%CPE
B	Toxicity	Positive	Positive	60%CPE	Toxicity	Positive	Positive	70%CPE	Toxicity	Positive	Positive	80%CPE
C	Toxicity	Positive	Positive	60%CPE	Toxicity	Positive	Positive	70%CPE	Toxicity	Positive	Positive	80%CPE
D	Toxicity	Positive	Positive	60%CPE	Toxicity	Positive	Positive	70%CPE	Toxicity	Positive	Positive	80%CPE
E	Toxicity	Positive	Positive	60%CPE	Toxicity	Positive	Positive	70%CPE	Toxicity	Positive	Positive	80%CPE
F	Toxicity	Positive	Positive	60%CPE	Toxicity	Positive	Positive	70%CPE	Toxicity	Positive	Positive	80%CPE
G	Negative	Negative	Negative	Negative	Toxicity	Toxicity	Toxicity	Toxicity	Positive	Positive	Positive	Positive
H	Negative	Negative	Negative	Negative	Blank	Blank	Toxicity	Toxicity	Positive	Positive	Positive	Positive

K	Sample 7				Sample 8				Sample 9			
	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160
	1	2	3	4	5	6	7	8	9	10	11	12
A	Toxicity	60%CPE	60%CPE	Positive	Toxicity	Positive	Positive	Positive	Toxicity	60%CPE	70%CPE	80%CPE

B	Toxicity	60%CPE	60%CPE	Positive	Toxicity	Positive	Positive	Positive	Toxicity	60%CPE	70%CPE	80%CPE
C	Toxicity	60%CPE	60%CPE	Positive	Toxicity	Positive	Positive	Positive	Toxicity	60%CPE	70%CPE	80%CPE
D	Toxicity	60%CPE	60%CPE	Positive	Toxicity	Positive	Positive	Positive	Toxicity	60%CPE	70%CPE	80%CPE
E	Toxicity	60%CPE	60%CPE	Positive	Toxicity	Positive	Positive	Positive	Toxicity	60%CPE	70%CPE	80%CPE
F	Toxicity	60%CPE	60%CPE	Positive	Toxicity	Positive	Positive	Positive	Toxicity	60%CPE	70%CPE	80%CPE
G	Negative	Negative	Negative	Negative	Toxicity	Toxicity	Toxicity	Toxicity	Positive	Positive	Positive	Positive
H	Negative	Negative	Negative	Negative	Toxicity	Toxicity	Toxicity	Toxicity	Positive	Positive	Positive	Positive

K	Sample 10				Sample 11				Sample 12			
	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160
	1	2	3	4	5	6	7	8	9	10	11	12
A	Toxicity	60%CPE	60%CPE	70%CPE	Toxicity	60%CPE	60%CPE	60%CPE	Toxicity	60%CPE	60%CPE	60%CPE
B	Toxicity	60%CPE	60%CPE	70%CPE	Toxicity	60%CPE	60%CPE	60%CPE	Toxicity	60%CPE	60%CPE	60%CPE
C	Toxicity	60%CPE	60%CPE	70%CPE	Toxicity	60%CPE	60%CPE	60%CPE	Toxicity	60%CPE	60%CPE	60%CPE
D	Toxicity	60%CPE	60%CPE	70%CPE	Toxicity	60%CPE	60%CPE	60%CPE	Toxicity	60%CPE	60%CPE	60%CPE
E	Toxicity	60%CPE	60%CPE	70%CPE	Toxicity	60%CPE	60%CPE	60%CPE	Toxicity	60%CPE	60%CPE	60%CPE
F	Toxicity	60%CPE	60%CPE	70%CPE	Toxicity	60%CPE	60%CPE	60%CPE	Toxicity	60%CPE	60%CPE	60%CPE
G	Negative	Negative	Negative	Negative	Toxicity	Toxicity	Toxicity	Toxicity	Positive	Positive	Positive	Positive
H	Negative	Negative	Negative	Negative	Toxicity	Toxicity	Toxicity	Toxicity	Positive	Positive	Positive	Positive

K	Sample 13				Sample 14				Sample 15			
	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160
	1	2	3	4	5	6	7	8	9	10	11	12
A	Toxicity	60%CPE	50%CPE	50%CPE	Toxicity	80%CPE	80%CPE	80%CPE	Toxicity	60%CPE	60%CPE	60%CPE
B	Toxicity	60%CPE	50%CPE	50%CPE	Toxicity	80%CPE	80%CPE	80%CPE	Toxicity	60%CPE	60%CPE	60%CPE
C	Toxicity	60%CPE	50%CPE	50%CPE	Toxicity	80%CPE	80%CPE	80%CPE	Toxicity	60%CPE	60%CPE	60%CPE
D	Toxicity	60%CPE	50%CPE	50%CPE	Toxicity	80%CPE	80%CPE	80%CPE	Toxicity	60%CPE	60%CPE	60%CPE
E	Toxicity	60%CPE	50%CPE	50%CPE	Toxicity	80%CPE	80%CPE	80%CPE	Toxicity	60%CPE	60%CPE	60%CPE
F	Toxicity	60%CPE	50%CPE	50%CPE	Toxicity	80%CPE	80%CPE	80%CPE	Toxicity	60%CPE	60%CPE	60%CPE

G	Negative	Negative	Negative	Negative	Toxicity	Toxicity	Toxicity	Toxicity	Positive	Positive	Positive	Positive
H	Negative	Negative	Negative	Negative	Toxicity	Toxicity	Toxicity	Toxicity	Positive	Positive	Positive	Positive

K	Sample 16				Sample 17				Sample 18			
	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160
	1	2	3	4	5	6	7	8	9	10	11	12
A	Toxicity	50%CPE	60%CPE	60%CPE	Toxicity	60%CPE	60%CPE	60%CPE	Positive	Positive	Positive	Positive
B	Toxicity	50%CPE	60%CPE	60%CPE	Toxicity	60%CPE	60%CPE	60%CPE	Positive	Positive	Positive	Positive
C	Toxicity	50%CPE	60%CPE	60%CPE	Toxicity	60%CPE	60%CPE	60%CPE	Positive	Positive	Positive	Positive
D	Toxicity	50%CPE	60%CPE	60%CPE	Toxicity	60%CPE	60%CPE	60%CPE	Positive	Positive	Positive	Positive
E	Toxicity	50%CPE	60%CPE	60%CPE	Toxicity	60%CPE	60%CPE	60%CPE	Positive	Positive	Positive	Positive
F	Toxicity	50%CPE	60%CPE	60%CPE	Toxicity	60%CPE	60%CPE	60%CPE	Positive	Positive	Positive	Positive
G	Negative	Negative	Negative	Negative	Toxicity	Toxicity	Toxicity	Toxicity	Positive	Positive	Positive	Positive
H	Negative	Negative	Negative	Negative	Toxicity	Toxicity	Toxicity	Toxicity	Positive	Positive	Positive	Positive

K	Sample 19				Sample 20				Cell culture media control			
	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160
	1	2	3	4	5	6	7	8	9	10	11	12
A	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Negative	Negative	Negative	Negative
B	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Negative	Negative	Negative	Negative
C	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Negative	Negative	Negative	Negative
D	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Negative	Negative	Negative	Negative
E	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Negative	Negative	Negative	Negative
F	Toxicity	Positive	Positive	Positive	Toxicity	Positive	Positive	Positive	Negative	Negative	Negative	Negative
G	Negative	Negative	Negative	Negative	Toxicity	Toxicity	Toxicity	Toxicity	Positive	Positive	Positive	Positive
H	Negative	Negative	Negative	Negative	Toxicity	Toxicity	Toxicity	Toxicity	Positive	Positive	Positive	Positive

(Part-4) ELISA

1-1:200, 1:400, 1:800 dilutions

	Samples M (1-8)						Samples K (1-8)					
	1:200		1:400		1:800		1:200		1:400		1:800	
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.124	0.128	0.097	0.094	0.075	0.070	0.124	0.126	0.079	0.082	0.071	0.073
B	0.079	0.077	0.063	0.066	0.060	0.055	0.056	0.057	0.054	0.055	0.056	0.054
C	0.085	0.090	0.068	0.074	0.065	0.060	0.072	0.065	0.057	0.059	0.055	0.059
D	0.072	0.065	0.063	0.057	0.057	0.054	0.116	0.115	0.076	0.076	0.067	0.070
E	0.089	0.083	0.066	0.068	0.059	0.058	0.112	0.115	0.081	0.075	0.065	0.068
F	0.067	0.065	0.058	0.055	0.055	0.053	0.082	0.087	0.066	0.067	0.060	0.063
G	0.081	0.083	0.071	0.072	0.068	0.061	0.061	0.064	0.055	0.056	0.055	0.061
H	0.058	0.054	0.055	0.051	0.054	0.050	0.054	0.060	0.052	0.054	0.055	0.060

	Samples M (1-8)						Samples K (1-8)					
	1:200		1:400		1:800		1:200		1:400		1:800	
	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	1	1	1	1	1	1	1	1	1	1
B	2	2	2	2	2	2	2	2	2	2	2	2
C	3	3	3	3	3	3	3	3	3	3	3	3
D	4	4	4	4	4	4	4	4	4	4	4	4
E	5	5	5	5	5	5	5	5	5	5	5	5
F	6	6	6	6	6	6	6	6	6	6	6	6
G	7	7	7	7	7	7	7	7	7	7	7	7
H	8	8	8	8	8	8	8	8	8	8	8	8

2-1:50, 1:100 dilutions

	Samples M (1-11)						Samples K (1-11)					
	1:50		1:100		1:50		1:50		1:100		1:50	
	1	2	3	4	5	6	7	8	9	10	11	12

A	0.151	0.128	0.093	0.092	0.060	0.066	0.141	0.138	0.113	0.112	0.059	0.055
B	0.057	0.060	0.054	0.051	0.233	0.244	0.049	0.048	0.052	0.051	0.116	0.139
C	0.090	0.081	0.072	0.064	0.120	0.131	0.058	0.057	0.054	0.054	0.055	0.053
D	0.061	0.058	0.057	0.051	0.046	0.046	0.083	0.086	0.070	0.070	0.050	0.049
E	0.071	0.073	0.063	0.063	0.059	0.056	0.153	0.175	0.122	0.133	0.053	0.051
F	0.066	0.067	0.056	0.055	0.146	0.144	0.078	0.087	0.070	0.072	0.089	0.095
G	0.073	0.070	0.062	0.062	0.093	0.095	0.056	0.064	0.053	0.058	0.051	0.053
H	0.056	0.054	0.054	0.052	0.046	0.046	0.072	0.071	0.059	0.061	0.053	0.050

	Samples M (1-11)						Samples K (1-11)					
	1:50		1:100		1:50		1:50		1:100		1:50	
	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	1	1	9	9	1	1	1	1	9	9
B	2	2	2	2	10	10	2	2	2	2	10	10
C	3	3	3	3	11	11	3	3	3	3	11	11
D	4	4	4	4	Blank	Blank	4	4	4	4	Blank	Blank
E	5	5	5	5	9 (1:100)	9 (1:100)	5	5	5	5	9 (1:100)	9 (1:100)
F	6	6	6	6	10(1:100)	10(1:100)	6	6	6	6	10(1:100)	10(1:100)
G	7	7	7	7	11(1:100)	11(1:100)	7	7	7	7	11(1:100)	11(1:100)
H	8	8	8	8	Blank	Blank	8	8	8	8	Blank	Blank

	Samples M (12-20)						Samples K (12-20)					
	1:50		1:100		1:50		1:50		1:100		1:50	
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.061	0.062	0.057	0.052	0.156	0.151	0.083	0.082	0.086	0.077	0.056	0.059
B	0.057	0.052	0.049	0.050	0.042	0.042	0.046	0.051	0.051	0.053	0.047	0.051
C	0.088	0.082	0.064	0.063	0.042	0.043	0.044	0.045	0.044	0.046	0.044	0.047
D	0.047	0.046	0.045	0.045	0.045	0.042	0.233	0.219	0.132	0.137	0.042	0.057
E	0.046	0.045	0.044	0.045	1.065	0.829	0.559	0.070	0.064	0.087	0.048	0.048
F	0.049	0.047	0.046	0.045	0.043	0.837	0.167	0.163	0.128	0.130	0.044	0.043
G	0.046	0.048	0.048	0.044	0.045	0.046	0.043	0.046	0.043	0.043	0.042	0.044
H	0.051	0.048	0.045	0.052	0.048	0.043	0.086	0.070	0.073	0.084	0.043	0.043

	Samples M (1-11)						Samples K (1-11)					
	1:50		1:100		1:50		1:50		1:100		1:50	
	1	2	3	4	5	6	7	8	9	10	11	12
A	12	12	12	12	20	20	12	12	12	12	20	20
B	13	13	13	13	Blank	Blank	13	13	13	13	Blank	Blank
C	14	14	14	14	Blank	Blank	14	14	14	14	Blank	Blank
D	15	15	15	15	Blank	Blank	15	15	15	15	Blank	Blank
E	16	16	16	16	**20** (1:100)	**20**(1:100)	**16**	16	16	16	20 (1:100)	20(1:100)
F	17	17	17	17	Blank	**Blank**	17	17	17	17	Blank	Blank
G	18	18	18	18	Blank	Blank	18	18	18	18	Blank	Blank
H	19	19	19	19	Blank	Blank	19	19	19	19	Blank	Blank

IFAT NT studies -

muIWV samples

M1

	1:20		**1:40**		**1:80**	
	Cells counted	**Virus+ve**	**Cells counted**	**Virus+ve**	**Cells counted**	**Virus+ve**
A	353	0	374	1	396	4
B	489	0	443	5	417	6
C	521	0	538	3	577	3
D	1487	0	1384	6	1443	8
E	455	0	421	2	637	11
F	589	0	546	7	573	9
Average	**649**	**0**	**617. 7**	**4**	**673.8**	**6.8**
Percentage	**0 %**		**0.65 %**		**1.01 %**	

Sample M-2

	1:20		1:40		1:80	
	Cells counted	**Virus+ve**	**Cells counted**	**Virus+ve**	**Cells counted**	**Virus+ve**
A	319	0	296	2	370	4
B	231	1	236	4	362	5
C	274	0	308	1	354	2
D	170	0	280	7	417	6
E	249	1	259	4	313	7
F	292	0	347	8	418	9
Average	**255.8**	**0.3**	**287.7**	**4.3**	**372.3**	**5.5**
Percentage	**0.13 %**		**1.51 %**		**1.48 %**	

Sample M-3

	1:20		1:40		1:80	
	Cells counted	**Virus+ve**	**Cells counted**	**Virus+ve**	**Cells counted**	**Virus+ve**
A	396	1	277	2	301	3
B	492	0	366	4	428	4
C	362	1	288	6	345	0
D	429	2	313	2	420	5
E	353	0	327	7	348	6
F	433	1	340	3	357	5
Average	**410.8**	**0.8**	**318.5**	**4**	**366.5**	**3.8**
Percentage	**0.20284 %**		**1.255887 %**		**1.04593 %**	

Sample M-4

	1:20		1:40		1:80	
	Cells counted	**Virus+ve**	**Cells counted**	**Virus+ve**	**Cells counted**	**Virus+ve**
A	318	0	372	1	405	2
B	420	0	342	0	351	3
C	357	0	301	2	374	1
D	330	0	362	1	410	6
E	272	0	285	3	449	5
F	381	0	316	2	426	8
Average	**346.3**	**0**	**329.7**	**1.5**	**402.5**	**4.2**
Percentage	**0 %**		**0.46%**		**1.04%**	

Sample M-5

	1:20		1:40		1:80	
	Cells counted	**Virus+ve**	**Cells counted**	**Virus+ve**	**Cells counted**	**Virus+ve**
A	365	0	366	2	415	3
B	361	1	387	1	359	2
C	373	0	349	3	388	5
D	370	0	324	1	355	4
E	428	2	413	4	352	6
F	352	0	346	2	281	4
Average	**374.8**	**0.5**	**364.2**	**2.17**	**358.3**	**4**
Percentage	**0.13 %**		**0.59%**		**1.12%**	

Sample M-11

	1:20		1:40		1:80	
	Cells counted	**Virus+ve**	**Cells counted**	**Virus+ve**	**Cells counted**	**Virus+ve**
A	199	0	305	0	684	0
B	582	0	242	0	819	1
C	280	0	349	1	413	0
D	340	0	920	0	645	0
E	370	0	273	0	511	0
F	636	0	860	0	572	13
Average	**401.2**	**0**	**491.5**	**0.17**	**607.3**	**2.3**
Percentage	**0 %**		**0.034 %**		**0.38 %**	

Controls

	Culture media with 1% FBS cells as a Control		Normal Salmon Plasma + Virus as a Control		Virus as a Control	
	Cells counted	Virus+ve	Cells counted	Virus+ve	Cells counted	Virus+ve
1	449	0	283	6	306	0
2	261	0	243	4	284	6
3	356	0	328	8	268	21
4	378	0	345	8	323	14
5	348	0	345	8	373	10
6	329	0	296	9	360	16
7	358	0	356	3	457	5
8	331	0	351	5	466	4
Average	**351.3**	**0**	**318.4**	**6.4**	**354.6**	**9.5**
Percentage	**0 %**		**2.00 %**		**2.68 %**	

DNA-Samples

Sample K-1

	1:20		1:40		1:80	
	Cells counted	Virus +ve	Cells counted	Virus +ve	Cells counted	Virus +ve
A	680	0	400	3	466	8
B	508	0	500	6	517	17
C	732	1	757	2	449	18
D	727	0	633	15	777	23
E	494	0	447	5	786	6
F	868	1	755	4	573	11
Average	**668.2**	**0.3**	**582**	**5.8**	**594.7**	**13.8**
Percentage	**0.05 %**		**1.0 %**		**2.3%**	

Sample K-2

	1:20		1:40		1:80	
	Cells counted	**Virus +ve**	**Cells counted**	**Virus +ve**	**Cells counted**	**Virus +ve**
A	393	0	293	2	341	4
B	381	1	331	5	328	7
C	348	0	443	4	357	9
D	284	2	309	3	284	6
E	297	0	268	6	324	4
F	284	1	326	7	341	11
Average	**331.2**	**0.7**	**328.3**	**4.5**	**329.2**	**6.8**
Percentage	**0.2 %**		**1.4 %**		**2.1%**	

Sample K-3

	1:20		1:40		1:80	
	Cells counted	**Virus +ve**	**Cells counted**	**Virus +ve**	**Cells counted**	**Virus +ve**
A	239	0	342	1	433	3
B	400	0	510	5	576	6
C	376	0	409	1	487	4
D	381	0	192	0	304	1
E	422	0	432	4	489	5
F	402	0	367	2	398	7
Average	**370**	**0**	**375.3**	**2.2**	**447.8**	**4.3**
Percentage	**0 %**		**0.6 %**		**1.0 %**	

Sample K-4

	1:20		1:40		1:80	
	Cells counted	**Virus+ve**	**Cells counted**	**Virus+ve**	**Cells counted**	**Virus+ve**
A	309	1	383	2	361	5
B	444	0	377	1	425	6
C	386	0	388	2	490	8
D	269	1	345	0	315	12
E	437	1	438	5	507	3
F	379	0	362	1	403	0
Average	**370.7**	**0.5**	**382.2**	**1.8**	**416.8**	**5.7**
Percentage	**0.1%**		**0.5 %**		**1.4 %**	

Sample K-5

	1:20		1:40		1:80	
	Cells counted	**Virus+ve**	**Cells counted**	**Virus+ve**	**Cells counted**	**Virus+ve**
A	438	0	406	4	322	8
B	193	0	446	2	353	12
C	408	1	464	5	417	9
D	413	0	378	3	350	13
E	401	0	325	7	259	8
F	342	0	361	5	401	6
Average	**365.8**	**0.2**	**396.7**	**4.3**	**350.3**	**9.3**
Percentage	**0.1**		**1.1 %**		**2.7 %**	

Sample K-11

	1:20		1:40		1:80	
	Cells counted	**Virus+ve**	**Cells counted**	**Virus+ve**	**Cells counted**	**Virus+ve**
A	1110	0	1040	0	995	0
B	716	0	1238	3	1007	4
C	785	2	914	0	1002	0
D	930	0	811	1	943	3
E	1158	0	844	0	988	0
F	1526	3	998	5	1214	4
Average	**1037.5**	**0.8**	**974.2**	**1.5**	**1024.8**	**1.8**
Percentage	**0.1 %**		**0.2 %**		**0.2 %**	

Controls

	Culture media with 1% FBS cells as a Control		Normal Salmon Plasma + Virus as a Control		Virus as a Control	
	Cells counted	**Virus+ve**	**Cells counted**	**Virus+ve**	**Cells counted**	**Virus+ve**
1	776	0	684	58	536	42
2	950	0	614	64	622	51
3	798	0	702	61	682	49
4	448	0	335	14	311	6
5	413	0	355	11	360	4
6	320	0	432	12	363	24
7	477	0	451	7	422	6
8	453	0	465	5	433	4
Average	**579.4**	**0**	**504.8**	**29**	**466.1**	**23.3**
Percentage	**0 %**		**5.7 %**		**5.0 %**	

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