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Aquatic medicine

PD-Vaccination - Effect of Different Vaccines for Induction of Circulating Antibodies

PD-vaksinering – effekt av ulike vaksiner for induksjon av sirkulerende antistoffer

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Content

Foreword

Pancreas Disease (PD) is a hot topic and an important disease in Atlantic salmon in the Norwegian salmon farming industry. The different effects of the vaccines against PD are not yet fully documented. We find this as an interesting topic with a potential for further research. We look at this task as an opportunity to specialize in pancreas disease, which is highly relevant for further careers in the salmon farming industry.

Summary

Title: PD-Vaccination – Effect of Different Vaccines for Induction of Circulating Antibodies

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In this thesis we have examined different PD-vaccines' capability to induce circulating and
neutralizing antibodies in Atlantic salmon. We have tried to compare the level of circulating
and neutralizing antibodies from fish vaccinated with the DNA-vaccine Clynav and the whole
virus inactivated vaccine AlphaJect Micro 1 PD, and the combination of these when injected
at the same time. To detect the presence and level of circulating antibodies in the plasma
samples, we used an indirect ELISA method. The results showed that the DNA vaccine
induced the highest level of circulating antibodies, but there was a variation within the group.
The AlphaJect Micro 1 PD vaccine gave a lower level of circulating antibodies, and with less
variation within the group. When the two vaccines were combined, immunized fish had a low
level of circulating antibodies. We also used a control group that was obtained from the field,
vaccinated with the inactivated whole virus vaccine named Aquavac PD7 (MSD), which was
also detected with circulating antibodies in the ELISA, but the level of antibodies in the group
was more dispersed than in the other groups. We cultured CHH1-cells and inoculated them
with SAV-3, and then detected CPE. We then performed a virus titration to find the $TCID_{50}$,
which was then used for the virus neutralization test. The virus neutralization test had invalid
results, with all wells containing plasma from the different vaccine groups having a positive
effect on CPE. Negative and positive control were as expected. Sources of error in the failed
virus neutralization test may include not optimal plasma storage that may have damaged the
antibodies, presence of some dead viruses in the supernatant used, and incorrect well row

readings that were then used to calculate TCID$_{50}$. Based on the outcome of our studies, we can only conclude that there is a presence of circulating antibodies in the different vaccines and in the combination of the two, at different levels. The DNA-vaccinated individuals had the highest level of circulating antibodies detected, while the combination-vaccinated individuals had the lowest level of circulating antibodies detected in the ELISA test. We can not say whether the antibodies are neutralizing or not, based on our failed virus neutralization result.

Definitions and abbreviations

APC	Antigen Presenting Cells
CMC	Cell Mediated Cytotoxicity
CMS	Cardiomyopathy Syndrome
CPE	Cytopathic Effect
CTL	Cytotoxic T-Lymphocytes
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
HRP	Horseradish Peroxidase
HSMI	Heart and Skeletal Muscle Inflammation
IPN	Infectious Pancreatic Necrosis
IWVV	Inactivated Whole Virus Vaccine
MHC	Major Histocompatibility Complex
PAMPs	Pathogen Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PD	Pancreas Disease
PRRs	Pattern Recognition Receptors
RPM	Revolutions Per Minute
SAV	Salmonid Alphavirus

SPDV	Salmonid Pancreas Disease Virus
TCID	Tissue Culture Infectious Dose
$TCID_{50}$	50% Tissue Culture Infectious Dose
TMB	Tetramethylbenzidine
VNT	Virus Neutralization Test

Introduction

In the Norwegian salmon farming industry, pancreas disease is a threat to fish welfare and the economic growth of the industry [1]. The disease is caused by salmonid alphavirus, often referred to as pancreas disease (PD), and is present at the west coast of Norway in the endemic PD-zone which extends from Jærens rev, Søre Revtangen to Skjemta, Flatanger [1]. Two subtypes of the virus are present in the PD-zone of Norway, where SAV-2 dominates in north from Hustadvika to Skjemta, Flatanger and SAV-3 in south from Hustadvika to Jærens rev, and this area defines the PD-zone [1]. Outside this zone (south and north), there is a control zone for SAV [1].

In 2014, SAV infections were noted on the list of infectious fish diseases by OIE [1-3]. The listing makes it possible for SAV-free countries to refuse import of salmon from areas that is not declared as SAV-free, regardless of free trade deals. In Norway SAV is listed as a list 3 disease, which means it is controlled and handled according to national standards [4].

According to the current regulations it is demanded to vaccinate all salmon and rainbow trout against PD before transfer to the sea in the northern area of the PD-zone (Fræna in the south) and a short distance inwards in the northern observation zone (Sømna in the north) [5]. There are different commercial vaccines available against PD in Norway, based on inactivated whole virus antigens and a DNA/plasmid vaccine. There are few studies that addressed elicited immune responses from DNA vaccine compared to inactivated whole virus vaccines (IWVV), that being antibody responses in general or neutralizing antibody responses in particular. Also, to which extent a combination of IWVV and DNA would be beneficial is not known. Therefore, the overall objective of this work is to assess the ability of the two different vaccine types to induce circulating and neutralizing antibodies in Atlantic salmon (*Salmo salar,* L.).

SAV

Salmon pancreas disease virus (SPDV) or salmonid alphavirus (SAV) causes pancreas disease (PD) in farmed Atlantic salmon (*Salmo salar* L.), first reported in 1976 in Scotland [6]. The official name of the virus is salmon pancreas disease virus (SPDV), and terms like Pancreas disease virus (PDV), or (Norwegian) salmonid alphavirus (subtype 3) [7] are also frequently used. SAV infection causes sleeping disease (SD) in rainbow trout (*Oncorhynchus mykiss*) in freshwater, reported and described in France [8]. The virus is termed Sleeping disease virus (SDV). By sequencing and comparing the complete genomes of SPDV and SDV, it has been demonstrated that both viruses are closely related genetically and are therefore generally referred to as salmonid alphavirus [9].

Properties, replication and transmission of the virus

SAV is a spherical and enveloped, 66nm in diameter, single-stranded, positive-sense RNA virus with a 11-12 kb genome, belonging to the genus Alphavirus in the family *Togaviridae* [3, 7]. The alphavirus is built up by an icosahedral nucleocapsid, which is enclosed by an envelope made by the previous host cell plasma membrane through the process of budding, and the envelope has the transmembrane glycoproteins E1 and E2 docked into the envelope as heterodimeric glycoprotein spikes [7, 10]. E1 and E2 are most likely interacting with the nucleocapsid by their C-terminal cytoplasmic region [11, 12]. E2 is essential for infection of a host cell and is responsible for receptor-binding, while E1 is responsible for fusion of the virus membrane with the host cell membrane and the process of internalization of the virus [13]. Presumably, E2 carries most of the neutralizing, protective epitopes, while E1 has conserved, cross-reactive epitopes of lesser importance for protection [14]. E1 and E2 are two out of a total of four structural capsid glycoproteins (E1, E2, E3 and 6K) encoded by the SAV genome [9, 14]. The genome has two open reading frames (ORFs)

which encodes a total of nine proteins, where the four structural glycoproteins and the capsid protein are coded by the 3´end and the 5´end encodes the four non-structural proteins (nsP1, nsP2, nsP3 and nsP4) [9, 14].

SAV replicates in the cytoplasm of the cells and is released by budding through the plasma membrane [7]. The whole process is first initiated by virus attachment to the host cell plasma membrane, via receptor binding by E2 and partly E1 to one or several cell surface proteins that function, with varying affinity, as functional receptors [15, 16]. By the process of clatherin-mediated endocytosis, Alphaviruses enter the cell and are then transported to the endosome [17, 18]. The viral spike proteins (E1) cause fusion of the viral membrane with the endosomal membrane due to the acidic environment within the endosome, and the nucleocapsid then enters the host cell cytosol where it is uncoated and the viral +ssRNA is then released and serves directly as mRNA for protein synthesis and later as template for -ssRNA generation [13, 16, 19]. From the 5´end of the genomic RNA (ORF-1), nsP1-4 are produced by autocleavage of the translated polyproteins [20]. At first the four nsPs form an early replication complex (due to an initial cleavage only between nsP3 and nsP4) which produces -ssRNA that, together with a late replication complex of the nsP1-4, functions as template for +ssRNA and 26S subgenomic RNA [21-23]. The 3´end of the genomic RNA ORF-2 is driven by the 26S subgenomic RNA promoter to encode the capsid and the four structural proteins (the two glycoproteins E1 and E2, and the two small peptides E3 and 6K) [16, 19]. Initially, all five structural proteins are translated as a 26S polyprotein, which are further processed individually by post-translational cleavage to their mature form [16]. The capsid protein is located at the N-terminus of the structural polyprotein and will at first, by its own serine protease activity at the C-terminal, co-translationally release itself from the polypeptide [24]. By a signal sequence the rest of the polyprotein (unprocessed pE2, E1 and 6K) will translocate into ER lumen where they will be released from each other by a host

signal peptidase [25]. In the ER lumen E1 and pE2 form heterodimers after folding and post-translational modification, and then they are transported to the plasma membrane via the secretory pathway where they are assembled into spikes and furin protease is cleaving pE2 into E2 and E3 proteins [26].

In the host cell cytoplasm a virus nucleocapsid core containing +ssRNA is assembled and is transported to the plasma membrane of the host cell where the virus structural proteins are present, and the alphavirus will then start a budding process from the infected host cell, spreading new mature enveloped virions [16].

Due to the important host cell receptor binding function of the E2 glycoprotein [27], it is reasonable to assume that genetic divergence of E2 has much to say for the replication rate of SAV as divergence may affect the affinity to host cell receptor(s). Virus virulence has been linked to amino acid residues in E2 of SAV [28], and a study with full-length genome sequencing of nine SAV-3 strains found a mean nucleotide diversity of 0.11%, with highest sequence divergence in 6K and E2 [29]. For the first time, this study reported that during natural infection with SAV, numerous defective viruses with genome deletions are generated with deletions aggregated in certain areas in the genome, so-called hot-spots [29]. As an explanation for generation of defective salmonid alpha viruses with genome deletions, imprecise homologous recombination was suggested [29]. In 2016 it was proven that SAV can change the genome by recombination, which results in new infective SAV variants or genomes with deletions that make the virus defect [30]. There are also findings to support that N-glycosylation consensus motifs of E1 and E2 are crucial for SAV virulence [31].

In general, Alphaviruses are transmitted by an insect vector, but this is not the case of SAV which mainly transmits horizontally in seawater from infected salmonids (SAV-2 freshwater variant in freshwater) [3, 32-34]. If vertical transmission happens at all, it is of minor importance [32]. Passive spread by the water current is suggested to cause SAV

transmission in seawater between farms and transport of salmon can contribute to spreading [35, 36].

The fish are most likely infected through the intestines or the gills [37]. At sea temperatures around 12-15 ºC the incubation time of SAV is estimated to be around seven to ten days [7]. Prior to occurrence of clinical signs and histopathological changes in target organs, there is viremia [7] which coincides with virus excretion [37, 38]. Studies suggest that virus excretion is due to natural excretion/secretion of SAV particles in faeces and mucus [39, 40]. During the viremia, SAV infect at around the same time kidney, spleen, heart and pancreas [41]. The virus can be detected in many tissues [38], but it is in the heart and pancreas that SAV replicates to high titre (highest viral load) [38, 41]. The primary target cell of SAV is not identified [37], but it is suggested that pancreas is a prime site of replication [7, 41, 42].

In water, the survival of SAV is dependent on the water temperature, where lower water temperatures increase its survival, but presence of organic materials in the water have a negative impact [37]. SAV has survived for more than two months at low temperatures in sterile sea water [43]. It is proven that SAV is sensitive to high temperatures (ensiling), low and high pH and commercial virucidal disinfectants [43, 44]. And standard disinfection methods of roe are considered sufficient to prevent SAV infection by surface contamination [32, 44].

Subtypes of the virus

Based on nucleotide sequences of E2 and nsP3, SAV is subdivided into six subtypes (SAV 1-6) [27, 45]. Despite the genetic subdivision, monoclonal antibodies are cross-reacting between the different subtypes of SAV, which indicates common antigenic epitopes and thus that the different subtypes are closely related serologically [46, 47].

SAV-1 cause pancreas disease in Scotland and Ireland, and the virus was first isolated from farmed Atlantic salmon in Ireland [33]. SAV-2 exist in two subgroups, a marine variant, and a freshwater variant [2, 3]. The marine variant cause pancreas disease in farmed Atlantic salmon in seawater in Scotland and Norway [3, 48], while the freshwater variant cause sleeping disease in Rainbow trout in freshwater as detected in France [8, 49], Germany [50], Scotland and England [51] and in some other mainland European countries [2]. SAV-3 has only been detected in Norway [52-54] and is therefore often referred to as Norwegian salmonid alphavirus, which causes PD in both Atlantic salmon and sea-reared Rainbow trout [52]. It was first isolated from PD-affected farmed Atlantic salmon in Norway in 1998 [55], but the disease was described in Norway as early as 1989 [56]. SAV-3 is closely related to SAV-1 (91.6%) and SAV-2 (92.9%) at nucleotide level [52]. SAV subtypes 4, 5 and 6 are detected in connection with PD-outbreaks in Ireland and Scotland, along with SAV-1 [27, 45]. A recent study is also suggesting the possibility of a SAV-7 subtype due to results from using nanopore sequencing for whole genome sequencing of a unique SAV strain isolated from ballan wrasse (*Labrus bergylta*), and is at the moment not showed to cause PD in salmonids [57].

Disease – outbreaks, signs and pathology

Clinical pancreas disease will occur only when a number of contributing factors interact, like stress, temperature and high viral infection pressure [7]. Clinical outbreaks are typically seen during Atlantic salmon smolts first year at sea [58] and a study suggested that post-smolt are more susceptible to infection with SAV-3 two weeks after seawater-transfer compared to nine weeks post transfer [59], but outbreaks have occurred at all stages of the marine production cycle [7]. Subclinical infections do occur [60, 61].

In PD outbreaks, the mortality rates range from 1 to 48% [7], but mortality rates up to 80% have been reported [62]. Studies suggest that the mortality rate is lower in clinical outbreaks caused by SAV-2 compared to SAV-3 infections [34, 63, 64]. And the proportion of fish that survives a clinical outbreak and fail to grow and become runts do vary from 1% to 31.5% [37, 65].

Typical clinical signs are sudden inappetence, lethargy and slow or circular swimming, increased faecal casts and increased mortality [7, 66]. Sudden death of apparently healthy fish due to exhaustion in combination with skeletal and cardiac damage have been reported [67].

Gross pathology findings at an early stage is typical presence of faecal casts and absence of food in the gut, and sometimes presence of petechiae at the surface of the pyloric caeca and the surrounding fat [7]. If pancreas does not regenerate, fish will, at a later stage, be observed with a low condition-factor and negligible body fat [7].

SAV cause tissue lesions, including hyaline degeneration of skeletal muscle cells in both red and white muscle fibers (also in oesophagus), degeneration and necrosis of cardiomyocytes (affecting both spongy and compact ventricular muscle and atrial muscle of the heart) and loss of pancreatic acinar cells due to pancreatic necrosis [6, 7, 68, 69]. The skeletal muscle lesions tend to appear three to four weeks after the appearance of the heart and pancreatic lesions, so skeletal muscle lesions can be the only lesions found in fish sampled at the late phase of the disease [7]. After some time, the tissue lesions will recover with varying degrees of success [7].

Pancreas disease is diagnosed based on clinical signs and histopathology with confirmation by virus isolation and cultivation (with cytopathic effect (CPE) development like small irregular foci of pyknotic vacuolated cells), RT-PCR and/or serology [7].

Three possible differential diagnosis to PD are HSMI, CMS and IPN [7]. HSMI is differentiated from PD by absence of pancreatic lesions and presence of myositis in red skeletal muscle fibers and heart lesions in the compact ventricle and epicardium. HSMI is typically occurring to salmon five to nine months after sea transfer [70]. CMS is differentiated from PD by absence of pancreatic lesions and myositis, and presence of heart lesions in the spongious ventricle and atrium. There is also often presence of liver pathology due to the chronic heart failure. CMS is occurring typical during the second year in sea [71]. IPN is differentiated from PD by the absence of heart and muscle lesions [72].

Vaccination

The principal of vaccination is to administer a killed or attenuated form of an infectious agents, or a component of a pathogen, which do not cause disease but elicits an immune response that provides protection against infections by the live pathogenic microbe [73]. They aim at stimulating the adaptive immune system to trigger a response against a pathogen, more specifically the immunogenic parts, which is defined structures of the pathogen [74]. The property of the pathogen and the vaccine itself impact on the success of controlling or eradicating the infectious diseases with vaccines. Most vaccines that are on the marked and being used today are inactivated, non-replicating vaccines, that work by inducing humoral immunity [75].

Antibodies play a crucial role in limiting or preventing the infection/infection success. They neutralize and clear pathogens before an infection is established or limit the spread of the virus locally or systemically in the host. An efficient vaccine stimulates the development of long-lived plasma cells that produce high-affinity antibodies as well as memory B-cells [73].

Administration of the vaccines to the fish differs for the different vaccines. They are either delivered by an intraperitoneal injection, by immersion (where the fish is placed in a vaccine solution), or by oral administration [74].

Immunity

The fish adaptive immune response includes both innate and adaptive immunity. Both are important for the defence against invading pathogens [76]. These responses can be profiled by measuring immunoglobulins (Ig), T-cell responses, and cytokine levels. The immune system of the fish is not understood in detail, but there is a difference in its efficiency and complexity compared to higher vertebrates [77].
Injecting an antigen can stimulate both humoral and cellular immune responses, where factors such as antigen dose, water temperature and stress caused by the handling can affect the outcome of vaccination.

The initial stage of the innate immune response involves involvement of pattern recognitions receptors (PRRs) which recognize and binds to pathogen associated molecular patterns (PAMPs). This leads to maturing of macrophages and dendritic cells, improving their antigen-presenting function. The result is expression of proinflammatory cytokines that attract more APC's. When the naïve B- and T-cell encounters the APC's, they mature into functional cells, plasma cells and activated T cells and a portion of long-lived memory cells, that are capable of recognizing antigens at later encounter. In fish, APCs have been identified that carry PRRs that possess the capacity to bind to different PAMPs. Different from what occurs in mammals where antigen-loaded APCs migrate to the lymph nodes, the APCs in fish migrates to the head-kidney and spleen [78].

Cells of the B lymphocyte lineage mature into plasma cells that secrete antibodies. The humoral responses are initiated by specific B cell recognition of antigen in secondary

lymphoid organs. T-independent antigens bind to membrane immunoglobulins on mature, native B cells, and this generates signals required for their proliferation and differentiation into plasma cells [73]. T-dependent antigens require presentation of antigen fragments on APCs via their MHC class II molecules that interact with the specific B cell receptor, and where accompanying T cells which bind internal parts of the same antigen deliver secondary signals to the B cell for final activation.

Cell-mediated immunity is when the defence against pathogens is mediated by T-cells. This immunity is involved in providing protection against intracellular and extracellular pathogens. This starts with naïve T-cells that are activated in the peripheral lymphoid organs, where antigens are presented by mature dendritic cells/APCs, either via MHC class I ($CD8^+$ cells) or II ($CD4^+$ cells). This induces biological responses in T-cells such as secretion of cytokines, proliferation, an increase in number of cells with an antigen-specific clone and differentiation of naïve cells into effector cells and memory lymphocytes.

As mentioned, there are two major types of T cells, $CD4^+$ and $CD8^+$, and these cells are central in cell mediated immune reactions. The $CD4^+$ T-cells are a key cell orchestrating a multitude of immune responses and involve production of cytokines that exert an effect on other immune cells and directs immune responses, including activation of B-cells (as mentioned) and activation of macrophages for killing of intracellular bacteria. $CD8^+$ have the capability to kill infected cells once activated and are therefore responsible for the extermination of intracellular pathogens, viruses and bacteria [73]. In other words, cell-mediated immunity is when the defence against pathogens is mediated by T-cells or where T-cells play a crucial role in the immune response. This immunity involves biological responses such as secretion of cytokines, cell proliferation, an increase in number of antigen-specific effector cells.

In summary, the adaptive cell-mediated cytotoxicity (CMC) is a mechanism where host immune cells recognize and kills virus-infected cells, and this requires that the cytotoxic T lymphocytes (CTLs) express key molecules. The main cytotoxic cells are natural killer (NK) cells and CTLs. NK cells do not need any further help to recognize the cell and destroy them, but the CTLs are MHC class I restricted [79]. CTLs bind to infected cells, i.e. cells that express parts of the pathogenic organism on their surface. To this day there are few studies addressing induction of virus-specific CMC responses in fish after injection of inactivated oil-adjuvanted viral vaccines but where such studies have been carried out, responses are low or absent post vaccination [79].

Adjuvants

Adding adjuvants to the vaccine preparation will provoke a stronger innate immune response, which leads to increased expression of costimulatory molecules and production of cytokines which again impact on induction of T-cell responses [73]. Adjuvants trigger a local inflammation. A great majority of the commercial vaccines in the aquaculture industry contains inactivated antigens formulated in oil-adjuvants [80]. How adjuvants work, in general, is poorly understood. It is a known fact that many of the adjuvants gives a depot effect at the site of inoculation. This results in a slow release of the antigens and sustained presentation of antigens to immunocompetent cells [81]. This is a common reaction when using oil-based adjuvants, and it is likely that this is of importance when it comes to induction of immunity for fish vaccines [74].

Most of the vaccines for salmonid fish are administered parenterally and formulated with an oil adjuvant to enhance and sustain the immunological response. It is necessary to use adjuvants to better involve the innate immune system and thus strengthen adaptive immune responses, particularly when the antigen itself triggers a poor response. This depot effect,

where the antigens are being retained at the site of injection, is believed to be a prerequisite for long-term protection [74].

DNA vaccine

As stated [75] DNA vaccines consist of a bacterial plasmid with a strong promoter, gene of interest, and a polyadenylation/transcriptional termination sequence. A viral promoter, immediate early gene promotor, and an enhancer of humane cytomegalovirus, are used because they drive the expression in a great variety of tissue. Plasmid-DNA also contains its own bacterial DNA-replication signals and can therefore be harvested in great quanta using bacteria performed by standard protocols. The DNA is purified, dissolved in saline water or phosphate buffered saline water and is administered by injections.

DNA vaccination is based on the administration of the gene encoding the vaccine antigen, rather than the antigen itself [82]. This means that the vaccine, that contains encoded immunogenic proteins, is injected into the muscle of the host where it enters the cells and initiate the synthesis of its polypeptide antigen from the plasmid vector [78]. Subsequent expression of the antigen by cells in the vaccinated host triggers the host immune system. A single intramuscular injection of microgram amounts of DNA is shown to induce rapid and long-lasting protection.

Inoculation of a plasmid containing complementary DNA (cDNA) encoding a protein antigen leads to humoral and cell-mediated immune responses to the antigen [73]. Further, plasmid DNA has adjuvant-like activities through C-G rich motifs in the backbone [83].

When the vaccine has been administrated the transcribed antigens are found in the cytosol and subsequently expressed on the cell surface in context of MHC molecules

depending on which cells serve as the "antigen-factory". DNA vaccines have the potential to elicit both humoral and cellular immune responses [78].

Inactivated whole virus vaccine

An inactivated (or killed) vaccine contains intact virus particles, bacteria, or other pathogens that are treated in a way that they are killed (incapable to replicate). This eliminates their capability to cause disease, but they still inhabit their immunogenicity or parts thereof [73]. Since the pathogen can no longer replicate, the antigens will be taken up by antigen-presenting cells, processed and subsequently presented in context of MHC class II, and this will stimulate a humoral immune response [78].

Available vaccines for SAV

There are a few commercial vaccines against PD available for use. Some are one-component formulations for active immunization against PD, while others are multicomponent vaccines. Pharmaq's PD vaccine, AlphaJect Micro 1 PD, and MSD Animal Health's Norvax Compact PD are both single-component vaccines. Aquavac PD7 includes an inactivated PD virus plus 6 other antigens. In addition to these vaccines a DNA vaccine, Clynav produced by Elanco Animal Health, is available for sale in Norway.

AlphaJect Micro 1PD

AlphaJect Micro 1PD (Pharmaq) is an inactivated whole virus vaccine formulated as a water-in-oil vaccine. It stimulates the development of immunity against pancreas disease. Immunity is induced by 516 day-degrees. It is administered intraperitoneal in the midline, approximately a pelvic sized distance form the basis of the pelvic fin [84].

Aquavac PD7 vet.

Aquavac PD 7 (MSD Animal Health) is an inactivated whole virus vaccine, formulated as a water-in-oil vaccine. It stimulates immunity against PD. Immunity is induced by 500 day-degrees. It is administered intraperitoneally in the midline, approximately a pelvic sized distance form the basis of the pelvic fin [85].

Clynav

Clynav is a DNA vaccine (Elanco) and stimulates immunity against PD. Protection is achieved within 399 day-degrees after vaccination. According to Felleskatalogen the duration of immunity is 1 year. It is administered intramuscularly in the epaxial muscle at a 90° angle to the skin surface [86].

Laboratory methods for detection of antibodies

There are several different methods for detection of antibodies in vaccinated animals/fish where ELISA and virus neutralization test (VNT) are used most frequently. In this study we have used ELISA and VNT, presented in the following paragraphs.

ELISA

To measure the level of antibodies in plasma we used the Enzyme-Linked Immunosorbent Assay (ELISA) method. The ELISA was developed by the research group of Peter Perlmann and Eva Engvall at Stockholm University in Sweden in the 1970s [87]. An ELISA works by quantifying the amount of antibody or antigen in a sample by reading an enzymatic reaction. To do this, the test determine with a spectrophotometer the rate at which the enzyme converts a clear substrate to a coloured product [73]. The results of the ELISA is

shown by optical density (OD). This means that the higher OD, the more light has been absorbed by the sample and this means that the sample contains a higher concentration of antibodies [88].

There are four main types of ELISA; direct, indirect, sandwich and competitive ELISAs. The direct ELISA is the simplest type of ELISA that only requires an antigen and an enzyme-conjugated antibody that are used to detect the antigen [89]. The indirect ELISA includes a conjugated secondary antibody that will bind to the host's antibody [90]. The most common version of ELISA is the sandwich assay [90]. The sandwich ELISA uses two different antibodies that will react to different epitopes of the antigen, and you start by coating the plates with antibodies, not antigen. It is essential that the two antibodies used, will bind to different and nonoverlapping determinants on the antigen, otherwise the second antibody will not be able to bind to the antigen. [91]. The competitive ELISA is also known as a blocking or inhibition ELISA and it is usually used to measure the amount of antigen in a sample, but it can also be used to measure the amount of antibodies. The sample antigen competes with a reference to bind to a limited amount of labelled antibodies in the wells and the results are shown as a weaker output signal with the spectrophotometer. The more antigen in the sample, the weaker the output signal is. [92]

We used the indirect ELISA method for this study. A defined concentration of antigens were added to the wells and were allowed to bind to the bottom of the well. Plasma from Atlantic salmon that contained an unknown level of antibodies against SAV-3 where then added to the wells and allowed to bind. The unbound antibodies were removed by washing and the secondary antibody, the enzyme linked, could then bind [91]. The most common used enzymes for the ELISAs are alkaline phosphatase and horseradish peroxidase [93]. According to a lecture in immunohistochemistry 5.10.2021 with Scientist Amr Ahmed Abdelrahim Gamil at NMBU, HRP substrates are more stable and more affordable to use than

alkaline phosphatase, but alkaline phosphatase gives a longer lasting signal between 24-48 hours and is more sensitive than the HRP. Since we were going to detect the ELISA results the same day, we used a HRP labelled mouse anti-salmonid antibody as secondary antibody. The secondary antibody must have the ability to bind to the primary antibody (species specific). After adding the HRP labelled secondary antibody, the plate was incubated in the dark for the colour change to develop before adding the blocking-buffer.

The virus neutralization test

To compare the presence and magnitude of functional antibodies that prevent infection, we used the virus neutralization test (VNT). This serological test is based on inhibition of a virus in a cell culture by the presence of neutralizing antibodies in serum or plasma. The virus neutralization test is more labour intensive than ELISA and takes more time, but the advantage of the VNT is that it detects functional antibodies after vaccination, unlike ELISA that only measures the amount of antibodies, but not if they play a role in protection or not. The VNT are performed in four steps: 1) Plasma dilution, 2) Mixed incubation with plasma and live virus particles [94], 3) Incubation of mix on a monolayer of cells that are permissive to the virus [95] and 4) Detection of absence/presence of cytopathic effect (CPE) by microscope. [94]. If the cells show indication of virus infection, i.e. CPE, the antibodies were not able to neutralize the virus and the test is read as negative. The test is positive if there is no sign of CPE, and thus the antibodies prevent infection [95]. The antibody titre is established by the reciprocal value of the highest dilution of serum or plasma that prevents infection of the cell culture [94].

Aim of the study

The overall aim of this study was to determine the ability of different PD vaccines to induce circulating, neutralizing antibodies in Atlantic salmon.

Separate goals were defined as:

i) compare level of neutralizing antibodies elicited by two different vaccine types, i.e. a DNA-vaccine/Clynav and inactivated whole-virus vaccines /AlphaJect Micro 1 PD, and Aquavac PD 7

ii) compare level of neutralizing antibodies elicited when monovalent DNA and monovalent IWVVs were combined

Material and methods

The fish material

Atlantic salmon (*Salmo salar* L.) raised in a laboratory aquarium, of approximately 65g weight, were vaccinated by intramuscular injection with Clynav®, 0.1 mL intramuscularly in the epaxial muscle below the dorsal fin (n=30) in freshwater (termed DNA). An equal group of fish were given AlphaJect Micro 1 PD (containing an inactivated whole virus antigen, based on SAV-3), intraperitoneally, 50 µL/fish (n=30, termed AJ). And one group of fish were given a combination of both AlphaJect Micro 1 PD (0.05 mL i.p.) and DNA Clynav vaccine at 0.1 mL i.m. (Comb group). A third group included Atlantic salmon kept in farming locations of the industry (approx. 75-80 g at vaccination) vaccinated with Aquavac PD7 (termed MSD), containing an inactivated whole virus antigen, based on SAV-1 under field conditions, 0.1mL/fish intraperitoneally (n=30, MSD group). Samples were drawn >1000-degree days post vaccination. Control fish were kept unvaccinated (Neg). The fish were transferred to sea water approximately two weeks after vaccination.

Plasma samples were collected at >450-degree days post vaccination for the DNA, AJ and Comb groups. Fish were anesthetized using Benzocaine (40 mg/L of water), and blood samples were drawn from the caudal vein using a vacutainer (2 mL), containing lithium-heparin. Blood samples were transferred to ice after collection and kept cold until centrifuged, done at 3500 rpm for ten minutes (at 4 ºC). The supernatant was aspirated following centrifugation, transferred to 1 mL Eppendorf tubes, and stored at -20-25 ºC until examined. Plasma samples in the MSD group were collected by vacutainer, centrifuged and storage were as described above.

Splitting and culturing of CHH1 cells

The cell line CHH1 was established from normal chondroblast cells from heart tissue of Chum salmon (*Oncorhynchus keta* W.). The cells were cultivated at 20 ºC in 75 cm^2 (medium) sterile plastic flasks containing L-15 medium (Leibovitz´s L-15 Medium, GlutaMAX^{TM} Supplement) supplemented with 10% FBS (Fetal Bovine Serum).

Good sterile technique was used while working with the culturing and splitting of the CHH1 cell line, using 70% ethanol as disinfection of the workplace, LAF bench, cell bottles and equipment. Cell culture medium (L-15), Trypsin and PBS de Boer were at room temperature when used and only one cell line (CHH1) was kept in the LAF bench at any time.

The CHH1 was removed from the 20 ºC incubator and inspected under microscope for morphology and confluency. The CHH1 cells grow in an adherent mode and can be split when they are above 80% confluent. The split ratio chosen was 1:3, where the one confluent cell culture bottle is split into three new bottles of the same size (75 cm^2).

A new cell culture medium bottle was pre-filled with 17 mL medium (L-15 + 10% FBS), to ensure optimal environment for the cell suspension immediately after the splitting of the cells. The total amount of medium in the bottle was 20 mL, when 3 mL cell suspension (from the split bottle) was added.

Using VacuSafe and sterile Pasteur pipettes to aspirate, we removed the old media from the confluent CHH1 cell culture bottle and washed two times with 8 mL PBS de Boer (buffer), gently adding PBS de Boer to the opposite side of the attached cell layer in the bottle. This was done to avoid distorting the cell layer. The bottle was then tilted to allow buffer to cover the cells. The PBS solution was aspirated between the two washings.

Then we added 1 mL Trypsin-EDTA to the newly washed confluent CHH1 cell culture bottle and tilted the bottle so the whole cell surface was covered. Trypsin-EDTA is an enzyme that will function as a dissociation reagent, splitting the cells from each other and

detach them from the bottom of the plastic bottle. The process of splitting with Trypsin-EDTA took around 20-30 seconds. The amount of time needed for cell splitting with Trypsin-EDTA are dependent on the cell line and batch used, and needs to be followed carefully. This process normally takes from half a minute to a few minutes, depending on the cell line used. We also pounded the flask lightly on the side to ensure that all the CHH1 cells loosened from the bottom of the flasks.

By using microscope, we checked that the cells, containing 1 mL Trypsin-EDTA, had detached from the bottle surface and added 8 mL L-15 medium to make a suspension. By pipetting up and down, we made sure that there were no clusters of cells. Then by use of a pipette, 3 mL of the suspension was transferred to a new 75 cm^2 cultivating bottle (pre-filled with 17 mL medium L-15 + 10% FBS). All the bottles were closed tightly before they were taken out of the LAF bench. The bottles were marked with cell type, passage number, split ratio 1:3, date and operator initials. The newly split cells were then put into the 20 ºC incubator, and kept there for approximately one week, followed by a new split at 1:3 ratio once a week. All important information of the splitting and cultivating of the cell line are noted and kept in the Cell Journal of the laboratory at NMBU.

When the procedure was finished, we turned off the VacuSafe and microscope, and disinfected the LAF bench and equipment with 70% ethanol.

Virus inoculation and harvesting

We started by inoculating the CHH1-cells with SAV-3 (strain H10, GenBank accession no. AY604236). This was done by thawing a frozen sample of SAV-3 (stored at -80 °C) and adding 300 µL of thawed virus sample into 25 mL of cell culture medium in a T75 flask. The culture medium contained 1% FBS and 50 mg/mL Gentamicin. The cells were inspected daily by light microscopy and incubated for 6 days when full CPE was observed.

Then the supernatant was decanted from the culture flask to a 50 mL tube. The tube was centrifuged at 2500 rpm for 10 minutes at 4 °C. The cleared supernatant was carefully transferred to a new 50 mL tube making sure none of the pellet was extracted. Then the old tube containing the pellet was discarded.

Titration/TCID$_{50}$ protocol

CHH1 cells were seeded in 96-well plates and waited for 10 days until the cells were at 90-100% confluence.

We proceeded to prepare the virus dilutions in a U bottom 1.4 mL tubes using L-15 media supplement with 1% FBS and 50 mg/mL gentamycin by using reversed pipetting making sure the wells were resuspended very well before going to the next dilution. Change of pipette tip between every step:

1. 1:10 – Added 80 µL of virus in 720 µL media, mixed thoroughly using 1 mL pipette.

2. 1:100 – Added 80 µL of 1:10 virus dilution in 720 µL media, mixed thoroughly using 1 mL pipette.

3. 1:1000 – Added 80 µL of 1:100 virus dilution in 720 µL media, mixed thoroughly using 1 mL pipette.

4. 1:10 000 – Added 80 µL of 1:1000 virus dilution in 720 µL media, mixed thoroughly using 1 mL pipette.

5. 1:100 000 – Added 80 µL of 1:10000 virus dilution in 720 µL media, mixed thoroughly using 1 mL pipette.

6. 1:1 000 000 – Added 80 µL of 1:100000 virus dilution in 720 µL media, mixed thoroughly using 1 mL pipette

7. 1:10 000 000 – Added 80 μL of 1:1000000 virus dilution in 720 μL media, mixed thoroughly using 1 mL pipette

8. 1:1 000 000 00 – Added 80 μL of 1:10000000 virus dilution in 720 μL media, mixed thoroughly using 1 mL pipette

The plate was emptied before adding the virus dilutions. Transferred 100 μL of each dilution to six parallel wells (columns 1-6, figure 1) of the 96-well plate containing CHH1 cells prepared in earlier steps using a multichannel pipette and making sure it is the same amount in each pipette. In the remaining six parallel wells (7-12) media were added without virus, as a negative control. After all the wells were filled in the 96 plate it was left in the incubator on 15°C for seven days. On the 7th day the plate was checked under the microscope to examine the CPE. The titre was read again under the light microscope on day 10. The results from the reading of the titre were then used to calculate the titre via Excel using the Kärbers method [96] of virus quantification.

Dilution	1	2	3	4	5	6	7	8	9	10	11	12
-1												
-2												
-3												
-4												
-5												
-6												
-7												
-8												

Figure 1 – The figure shows the wells where blue is the different virus dilutions, and the white is the negative control.

ELISA

We used ELISA plates containing 96 wells. We analysed 15 samples per plate and each sample had three dilutions and two parallels. Six of the wells were used for controls; one positive (AlphaJect micro1 PD vaccinated Atlantic salmon), one negative (plasma from unvaccinated Atlantic salmon) and one blank (only NaCl).

	1	2	3	4	5	6	7	8	9	10	11	12
	Sample 1			**Sample 2**			**Sample 3**			**Sample 4**		
A	1:100	1:200	1:400	1:100	1:200	1:400	1:100	1:200	1:400	1:100	1:200	1:400
B	1:100	1:200	1:400	1:100	1:200	1:400	1:100	1:200	1:400	1:100	1:200	1:400
	Sample 5			**Sample 6**			**Sample 7**			**Sample 8**		
C	1:100	1:200	1:400	1:100	1:200	1:400	1:100	1:200	1:400	1:100	1:200	1:400
D	1:100	1:200	1:400	1:100	1:200	1:400	1:100	1:200	1:400	1:100	1:200	1:400
	Sample 9			**Sample 10**			**Sample 11**			**Sample 12**		
E	1:100	1:200	1:400	1:100	1:200	1:400	1:100	1:200	1:400	1:100	1:200	1:400
F	1:100	1:200	1:400	1:100	1:200	1:400	1:100	1:200	1:400	1:100	1:200	1:400
	Sample 13			**Sample 14**			**Sample 15**					
G	1:100	1:200	1:400	1:100	1:200	1:400	1:100	1:200	1:400	Neg	Pos	Blank
H	1:100	1:200	1:400	1:100	1:200	1:400	1:100	1:200	1:400	Neg	Pos	Blank

Figure 2 – The figure shows the dilutions and parallels per sample on a plate.

Plasma from Atlantic salmon sampled from the different vaccine groups vaccinated with Alpha Ject micro1 PD, Clynav®, Aquavac PD 7, and a combination of Alpha Ject Micro 1 and Clynav were tested.

We had the buffers ready before we started, and these are the reagents that we used:

Coating buffer (500 mL):

Bicarbonate buffers ($0,785$g Na_2CO_3 + $1,465$g $NaHCO_3$ + distilled water). We added water up to total volume 500 mL. Ph adjustment is not necessary. The buffer is stable for at least two weeks when stored at 4°C.

10 x Phosphate buffered saline (PBS):

$1,6$g NaH_2PO_4 X H_2O

$9,8$g HPO_4 x $2H_2O$

81g NaCl

Distilled water is added up to total volume of 1 L.

Washing buffer (PBS/T):

100 mL 10X PBS

900 mL water

$0,5$ mL Tween 20 (0.05 %)

Blocking buffer:

5 % fat free dry milk in PBS

$2,5$g fat free dry milk

PBS/T is added up to a total volume of 50 mL.

Diluent buffer:

1% fat free dry milk in PBS

$0,5$g fat free dry milk

PBS/T is added up to a total volume of 50 mL.

Substrate solution: TMB ELISA substrate (Highest Sensitivity) from abcam (ab171522)

Stop solution: 1M HCL

Procedure

First, we coated the ELISA template wells with 10^5 TCID$_{50}$/mL SAV-3 with coating buffer. We used a dilution of 1/1000 of the antigen in the 96-well. To simplify the calculations, we used 100 wells instead of 96 and each well was filled with 100 µL: 100µL x 100 wells = 1000 µL = 10 mL. We added 100µL per well of PD virus and incubated the plates overnight at 4 ºC.

The next day we washed the plates with 250µL washing buffer per well, three times, using an ELISA plate washing machine. Before we washed the plates, we first used a test plate to check that all the wells got cleaned well and that there was used equal amount of water and detergent per well. Then we added 250µL of blocking buffer to each well and incubated the plate at room temperature for two hours.

After two hours of incubation, wells were washed three times, and at this time plasma dilutions were made. We added 600 µL diluent buffer to each well and then added, per sample, 6 µL (1:100) x 2, 3 µL (1:200) x 2 and 1,5 µL (1:400) x 2 plasma per sample, a total of two wells per dilution and a total of six wells overall per sample (see fig. 2). We used one positive, one negative and one blank control, made by 600 µL diluent buffer and 6 µL plasma. The positive control was plasma from vaccinated Atlantic salmon and the negative control was from non-vaccinated Atlantic salmon. The blank control contained NaCl only. We then incubated the plates in room temperature for one hour.

After one hour we washed the plates five times and added 70µL mouse anti salmonid HRP (horseradish peroxidase) labelled antibody at a dilution of 1:300 in diluent buffer for one

hour. We then washed the plates five times and added 70µL of the substrate solution. We incubated the plates for 15 minutes in the dark, at room temperature. Plates were not washed at this stage and 70µL of the stop solution was added to each well. Absorbance was read at 450 nm using a spectrophotometer.

Virus neutralization test

Procedure

We diluted the plasma samples (1:2, 1:4, 1:8 and 1:16) with cell culture medium without FBS stored at 4ºC. We then mixed each series of diluted plasma with SAV-3 1:1 giving a final virus concentration of 100 $TCID_{50}$ virus particles per well (see appendix) and incubated the mixture for one hour at room temperature. The plates were coated with CHH1-cells beforehand, with 75 % confluency. After incubation the SAV-3/plasma mix, we inoculated the plates with 100 µL of mixed samples per well in six parallels. Eight wells were used for positive control, eight wells for negative control and eight wells for normal serum from unvaccinated fish. The plates were put in an incubator set at 15 ºC. Seven days later we evaluated the CPE by microscopy.

	Sample 1				Sample 2				Sample 3			
A	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16
B	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16
C	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16
D	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16
E	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16
F	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16
G	Neg	Neg	Neg	Neg	NS	NS	NS	NS	Pos	Pos	Pos	Pos
H	Neg	Neg	Neg	Neg	NS	NS	NS	NS	Pos	Pos	Pos	Pos

Figure 3 – The figure shows the dilutions and parallels per sample used in the virus neutralization test.

Results

ELISA

All vaccine groups had a significant increase in antibody level compared to non-vaccinated controls (p<0.001). The Combination group at 1:100 dilution (p=0.190) was nearly equal to the control. At higher dilutions of the Combination group they differed from the control, p=0.041 for 1:200 and 1:400 dilutions (lower standard error). The average OD_{450} for the different vaccine groups and dilutions are shown in table 1.

	OD 1:100		OD 1:200		OD 1:400	
	Mean	Std. err.	Mean	Std. err	Mean	Std. err
Alfa Ject Micro 1	0.50	0.04	0.35	0.02	0.21	0.01
Combination	0.32	0.06	0.25	0.04	0.14	0.02
DNA	0.83	0.05	0.75	0.03	0.67	0.03
Aquavac PD7/MSD	1.95	0.13	1.74	0.11	1.34	0.11
Negative	0.06	0.01	0.06	0.01	0.06	0.01
Positive	1.22	0.14	1.22	0.14	1.22	0.14

Table 1

A graphic presentation of the ELISA results are shown for the three different dilutions (1:100, 1:200, 1:400) vaccinated with the different vaccine preparations are also shown in figure 4. As seen from table 1 and figure 4, the Aquavac PD 7 vaccinated fish (MSD group) have the highest circulating antibody levels (OD of 1.95±0.13 (±SE), 1.74±0.11 and 1.34±0.11, at 1:100, 1:200 and 1:400 dilution, respectively). At the same time there is more variation between fish in this group (higher SD, table 1 and fig. 4).

The DNA group has the second highest level of circulating antibodies post vaccination, average OD range from 0.83±0.05 (1:100) to 0.67±0.03 (1:400, table 1) but

significantly lower than MSD for all dilutions, p=0.0001 (Kruskal Wallis equality-of-populations rank test). Then the AJ group has significantly (p=0.0001, Kruskal Wallis equality-of-populations rank test) lower average OD_{450} values than the DNA and MSD groups (table 1). Finally, the mean OD_{450} in the Comb group was significantly lower than AJ and DNA groups (p<0.001 for 1:100, 1:200 and 1:400, respectively for both comparisons, Kruskal Wallis equality-of-populations rank test). Table 1 and figure 4 also includes the results for a positive and negative (non-vaccinated) controls.
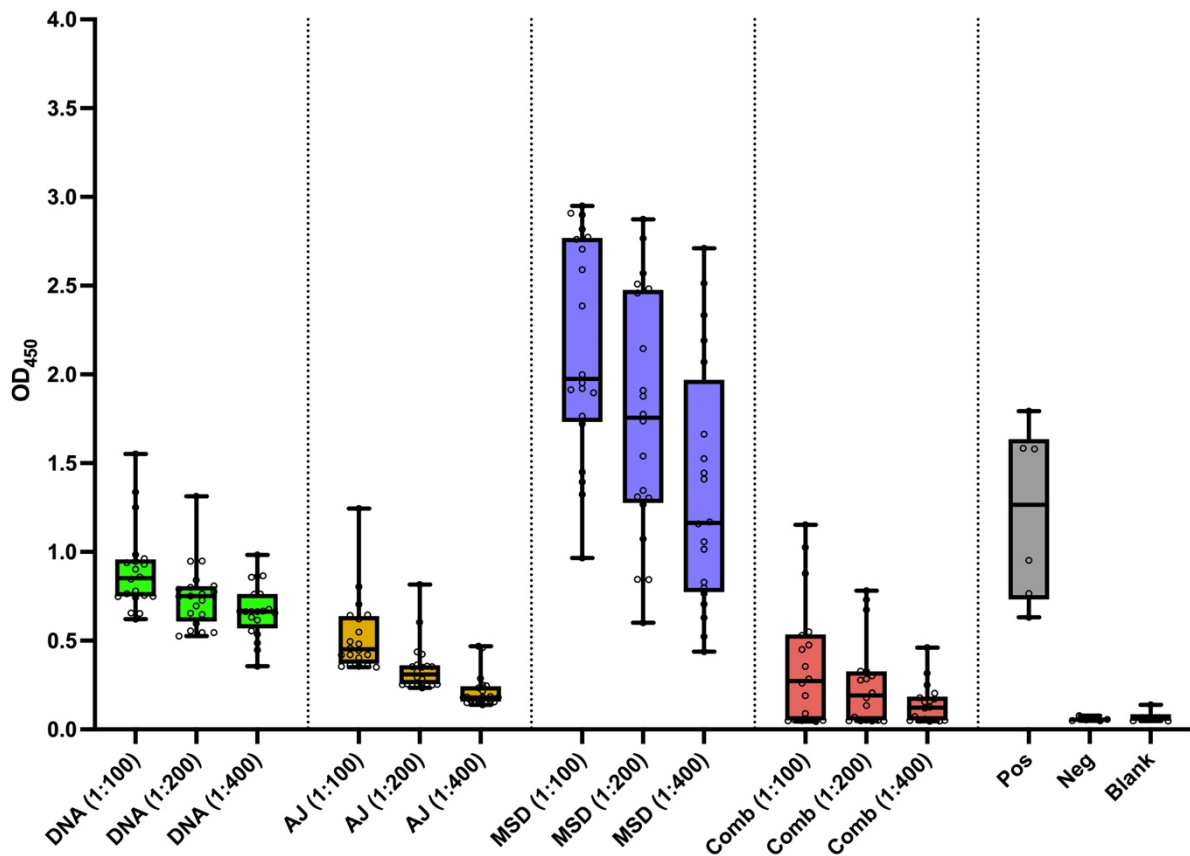
Figure 4 – The results are here represented in a box plot and it shows the OD_{450} of the different vaccines with the dilutions 1:100, 1:200 and 1:400. The boundaries of the box show the upper and lower quartile which means that half of the results are located inside the box. The line inside the box is the median. The end marking of the lines(whiskers) is the range of variation and the plots on the outside of this marking are outliners. The Y-axis shows the level of optical density at 450 nanometer absorbance. The X-axis shows the different groups of plasma samples and the different dilutions within each group.

The distribution in OD_{450} values for different vaccines is shown in figure 5-8, and graphed as histograms where the OD value is shown on the x-axis and the relative distribution (in percent) on the y-axis.
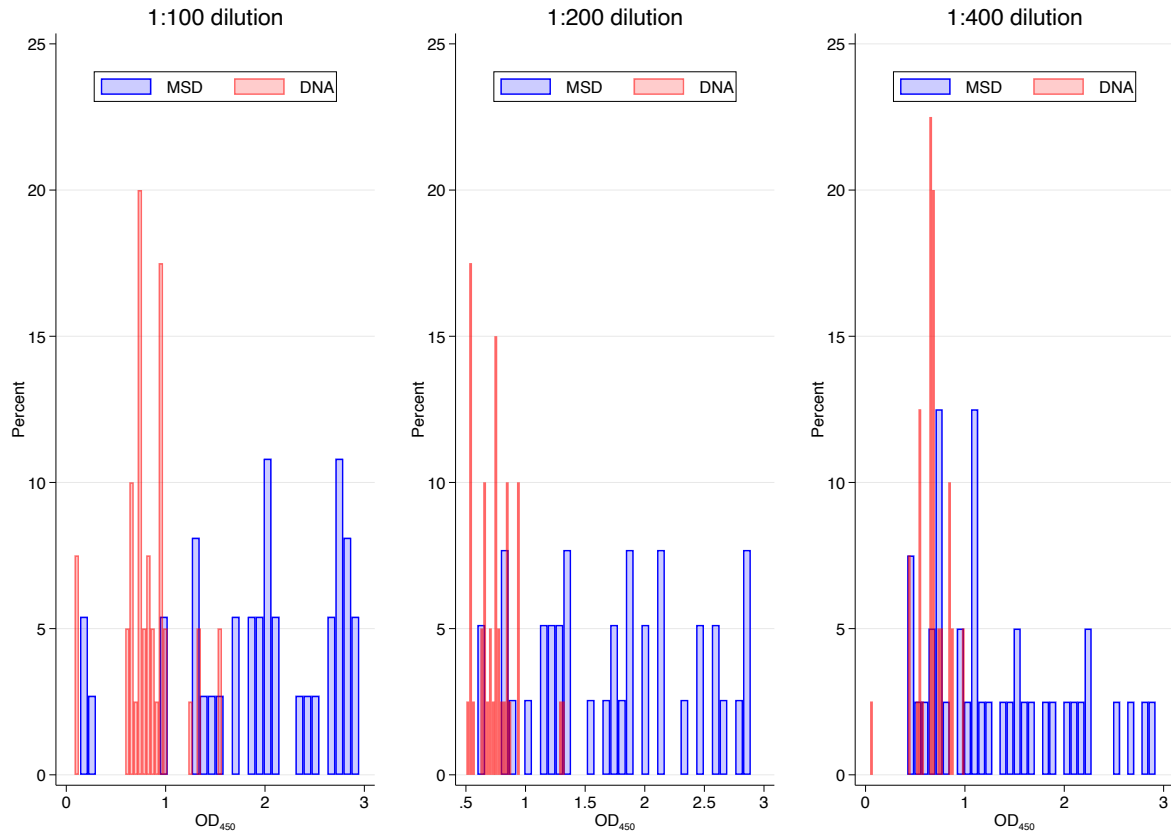
Figure 5 - Histograms showing distribution of OD values (x-axis) and percentage on y-axis for MSD and DNA vaccines at different dilutions.

There is a distinct separation in distribution of OD-values between MSD and DNA vaccinated groups (fig. 5), also reflected in the average OD-values, seen in table 1. The percent reduction in OD-values over the dilution series is 33.8% for MSD but only 19.3% for the DNA group (see table 1), which is also reflected in figure 4 showing a higher degree of overlap at 1:400 than for less diluted groups.
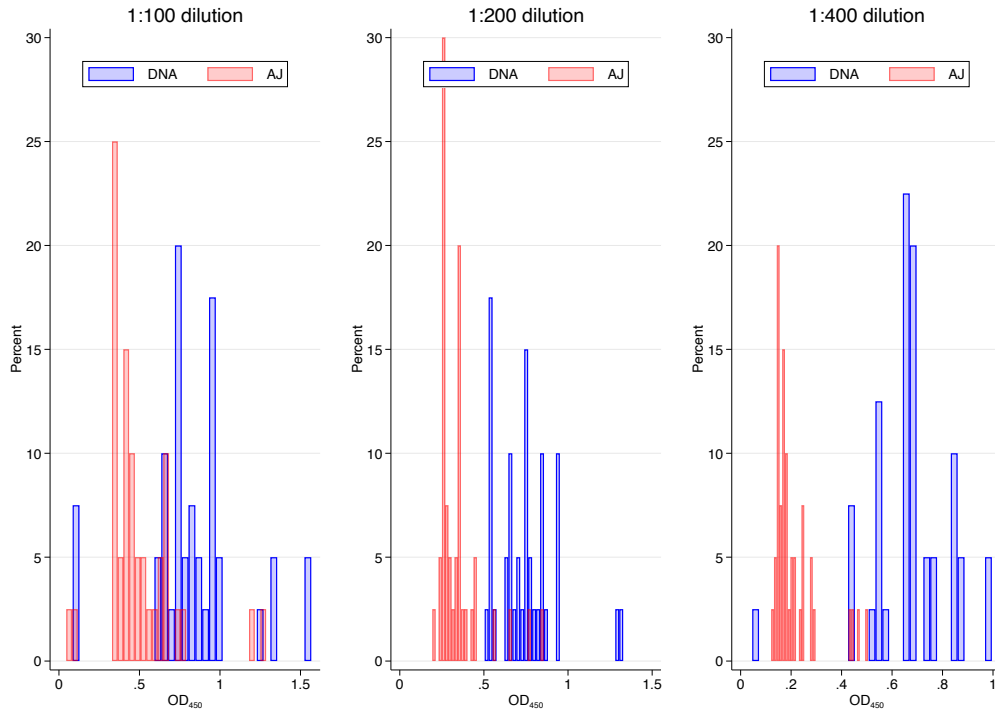
Figure 6 - Histograms showing distribution of OD values (x-axis) and percentage on y-axis for DNA and AJ vaccine groups at different dilutions as shown.

The overlap in OD-values for the two vaccine groups, DNA and AJ, is reduced with increasing dilution (fig. 6). While the reduction in OD-value over the series of dilutions is 19.3% for the DNA group, average OD-values for AJ are reduced by 56.9% with increasing dilution (0.497 to 0.213 for 1:100 and 1:400, respectively).
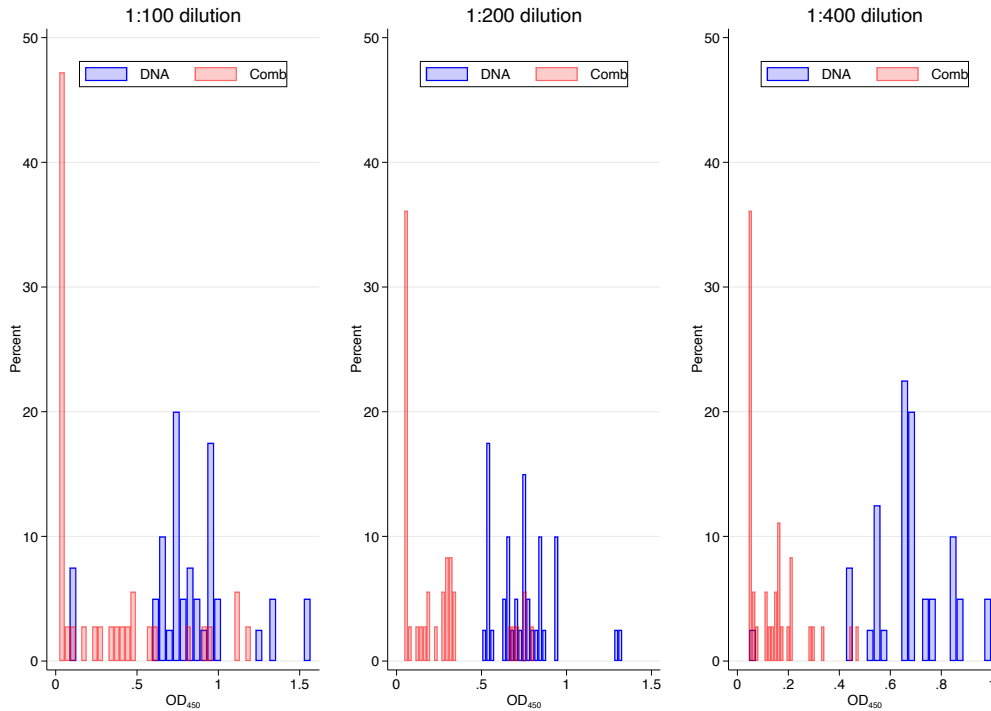
Figure 7 - Histograms showing distribution of OD values (x-axis) and percentage on y-axis for DNA and Comb vaccine groups at different dilutions as shown.
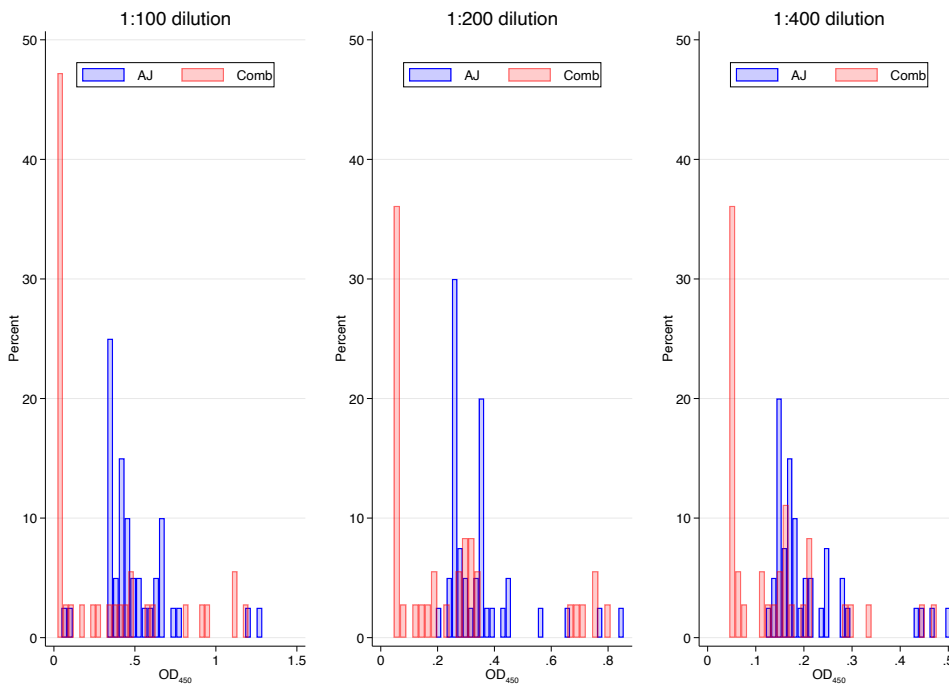
Figure 8 - Histograms showing distribution of OD values (x-axis) and percentage on y-axis for AJ and Comb vaccine groups at different dilutions as shown.

Comparing the degree of OD-value overlap for DNA/Comb (fig. 7) and AJ/Comb (fig. 8) there is separation between DNA and Comb with increasing dilutions while for AJ and Comb the separation between the two groups is less obvious. This aligns with the finding that OD values for the DNA group is reduced 19.3% and 55% for the Comb group, which means that AJ and Comb are comparable, 56.9% and 55%, respectively.

The statistical comparisons first included a calculation for normal distribution for the ELISA OD_{450} values obtained (see appendix) which showed a non-normal distribution which is the basis for using a the Kruskal-Wallis test.

Virus harvest and titration ($TCID_{50}$)

After inoculating the CHH1-cells with SAV-3, we could detect a full cytopathic effect (CPE) within six days, as shown by figure 9. The CPE present is seen as apoptotic cells, cell dissolution, vacuoles and increased space between the cells. The SAV-3 strain inoculated in six parallel wells with a dilution factor 10 between the eight rows, in a 96-well plate, had 100% CPE up to and including row 5 at day seven and ten. There were in total 12 positive CPE wells in row 5 and the other rows below (shown in figure 10). By using Kärbers method of virus quantification (see appendix), the virus titre was calculated to be $10^{7,5}$ $TCID_{50}$/mL.

Day 1	Day 3
A small number of cells showing cytopathic effect. The density of the cells is high.	Some apoptotic bodies are present. A few cells show signs of CPE.
Day 4	Day 6
CPE is visible in foci in the cell monolayer affecting approximately 5-10 % of the cells.	Cell culture with 70-80 % CPE. The density of the cells is lower, the cell debris is present. Apoptosis and vacuoles are present.

Figure 9 – This figure shows the development of CPE over six days after inoculating the CHH1-cells with SAV-3.

	1	2	3	4	5	6	7	8	9	10	9	11	12
A/-1	+	+	+	+	+	+	-	-	-	-	-	-	-
B/-2	+	+	+	+	+	+	-	-	-	-	-	-	-
C/-3	+	+	+	+	+	+	-	-	-	-	-	-	-
D/-4	+	+	+	+	+	+	-	-	-	-	-	-	-
E/-5	+	+	+	+	+	+	-	-	-	-	-	-	-
F/-6	+	+	+	+	+	-	-	-	-	-	-	-	-
G/-7	+	-	-	-	-	-	-	-	-	-	-	-	-
H/-8	-	-	-	-	-	-	-	-	-	-	-	-	-

Figure 10 - CPE registered (day 7 and 10 post SAV-3 inoculation) in the virus-inoculated CHH1 cells (96-well plate), 48 wells used (6 columns) for titration and column 7 to 12 were negative control only containing cell medium. With different dilutions (row A-H) in 6 parallel wells (columns 1-6). The last dilution series with 100% positive CPE (+) is row E/-5. The amount of remaining positive wells after and including the 100% positive CPE row is 12.

Virus neutralization test

At day seven post virus inoculation, the virus neutralization test showed typical SAV-induced cytopathic effect (CPE) in all the samples at all dilutions, for all vaccine groups, including the Comb group (figure 11). The negative controls showed no CPE and the positive controls showed CPE, as expected.

DNA Clynav Plate 1

	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16
	1	2	3	4	5	6	7	8	9	10	11	12
A	+	+	+	+	+	+	+	+	+	+	+	+
B	+	+	+	+	+	+	+	+	+	+	+	+
C	+	+	+	+	+	+	+	+	+	+	+	+
D	+	+	+	+	+	+	+	+	+	+	+	+
E	+	+	+	+	+	+	+	+	+	+	+	+
F	+	+	+	+	+	+	+	+	+	+	+	+
G	Neg	Neg	Neg	Neg	NS •	NS •	NS •	NS	Pos	Pos	Pos	Pos
H	Neg	Neg	Neg	Neg	NS •	NS •	NS	NS	Pos	Pos	Pos	Pos

DNA Clynav Plate 2

	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16
	1	2	3	4	5	6	7	8	9	10	11	12
A	+	+	+	+	+	+	+	+	+	+	+	+
B	+	+	+	+	+	+	+	+	+	+	+	+
C	+	+	+	+	+	+	+	+	+	+	+	+
D	+	+	+	+	+	+	+	+	+	+	+	+
E	+	+	+	+	+	+	+	+	+	+	+	+
F	+	+	+	+	+	+	+	+	+	+	+	+
G	Neg	Neg	Neg	Neg	NS •	NS •	NS	NS	Pos	Pos	Pos	Pos
H	Neg	Neg	Neg	Neg	NS •	NS •	NS	NS	Pos	Pos	Pos	Pos

Alpha Ject micro 1 Plate 1

	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16
	1	2	3	4	5	6	7	8	9	10	11	12
A	+	+	+	+	+	+	+	+	+	+	+	+
B	+	+	+	+	+	+	+	+	+	+	+	+
C	+	+	+	+	+	+	+	+	+	+	+	+
D	+	+	+	+	+	+	+	+	+	+	+	+
E	+	+	+	+	+	+	+	+	+	+	+	+
F	+	+	+	+	+	+	+	+	+	+	+	+
G	Neg	Neg	Neg	Neg	NS •	NS	NS	NS	Pos	Pos	Pos	Pos
H	Neg	Neg	Neg	Neg	NS •	NS	NS	NS	Pos	Pos	Pos	Pos

Alpha Ject micro 1 Plate 2

	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16
	1	2	3	4	5	6	7	8	9	10	11	12
A	+	+	+	+	+	+	+	+	+	+	+	+
B	+	+	+	+	+	+	+	+	+	+	+	+
C	+	+	+	+	+	+	+	+	+	+	+	+
D	+	+	+	+	+	+	+	+	+	+	+	+
E	+	+	+	+	+	+	+	+	+	+	+	+
F	+	+	+	+	+	+	+	+	+	+	+	+
G	Neg	Neg	Neg	Neg	NS •	NS	NS	NS	Pos	Pos	Pos	Pos
H	Neg	Neg	Neg	Neg	NS •	NS	NS	NS	Pos	Pos	Pos	Pos

Combination Plate 1

	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16
	1	2	3	4	5	6	7	8	9	10	11	12
A	+	+	+	+	+	+	+	+	+	+	+	+
B	+	+	+	+	+	+	+	+	+	+	+	+
C	+	+	+	+	+	+	+	+	+	+	+	+
D	+	+	+	+	+	+	+	+	+	+	+	+
E	+	+	+	+	+	+	+	+	+	+	+	+
F	+	+	+	+	+	+	+	+	+	+	+	+
G	Neg	Neg	Neg	Neg	NS •	NS •	NS	NS	Pos	Pos	Pos	Pos
H	Neg	Neg	Neg	Neg	NS •	NS •	NS	NS	Pos	Pos	Pos	Pos

Combination Plate 2

	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16
	1	2	3	4	5	6	7	8	9	10	11	12
A	+	+	+	+	+	+	+	+	+	+	+	+
B	+	+	+	+	+	+	+	+	+	+	+	+
C	+	+	+	+	+	+	+	+	+	+	+	+
D	+	+	+	+	+	+	+	+	+	+	+	+
E	+	+	+	+	+	+	+	+	+	+	+	+
F	+	+	+	+	+	+	+	+	+	+	+	+
G	Neg	Neg	Neg	Neg	NS •	NS •	NS •	NS	Pos	Pos	Pos	Pos
H	Neg	Neg	Neg	Neg	NS •	NS •	NS	NS	Pos	Pos	Pos	Pos

MSD Plate 1

	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16
	1	2	3	4	5	6	7	8	9	10	11	12
A	+	+	+	+	+	+	+	+	+	+	+	+
B	+	+	+	+	+	+	+	+	+	+	+	+
C	+	+	+	+	+	+	+	+	+	+	+	+
D	+	+	+	+	+	+	+	+	+	+	+	+
E	+	+	+	+	+	+	+		+	+	+	+
F	(gray)	+	+	+	+	+	+	(gray)	+	+	+	+
G	Neg	Neg	Neg	Neg	NS •	NS •	NS	NS	Pos	Pos	Pos	Pos
H	Neg	Neg	Neg	Neg	NS •	NS •	NS	NS	Pos	Pos	Pos	Pos

MSD plate 2

	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16
	1	2	3	4	5	6	7	8	9	10	11	12
A	+	+	+	+	+	+	+	+	+	+	+	+
B	+	+	+	+	+	+	+	+	+	+	+	+
C	+	+	+	+	+	+	+	+	+	+	+	+
D	+	+	+	+	+	+	+	+	+	+	+	+
E	+	+	+	+	+	+	+		+	+	+	+
F	+	+	+	+	+	+	+	(gray)	+	+	+	+
G	Neg	Neg	Neg	Neg	NS •	NS •	NS	NS •	Pos	Pos	Pos	Pos
H	Neg	Neg	Neg	Neg	NS •	NS •	NS	NS	Pos	Pos	Pos	Pos

MSD plate 3

	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16
	1	2	3	4	5	6	7	8	9	10	11	12
A	+	+	+	+	+	+	+	+	+	+	+	+
B	+	+	+	+	+	+	+	+	+	+	+	+
C	+	+	+	+	+	+	+	+	+	+	+	+
D	+	+	+	+	+	+	+	+	+	+	+	+
E	+	+	+	+	+	+	+	+	+	+	+	+
F	+	+	+	+	+	+	+	+	+	+	+	+
G	Neg	Neg	Neg	Neg	NS •	NS •	NS •	NS	Pos	Pos	Pos	Pos
H	Neg	Neg	Neg	Neg	NS •	NS •	NS	NS	Pos	Pos	Pos	Pos

Figure 11 – The virus neutralization test results are shown for all the different vaccine groups. Every plate contains 3 samples at dilutions 1:2, 1:4, 1:8 and 1:16, and in 6 parallels (A-F). Every plate has negative controls, normal serum controls and positive controls. As shown in the figure, all wells (except negative controls) are positive for CPE (+). Some of the wells (MSD plate 1 and 2) contained too little plasma, marked in gray. Some of the normal serum wells showed signs of toxicity (marked as red dots). Positive controls turned out as expected.

Discussion

ELISA results

Fish vaccinated with Aquavac PD7 (SAV-1) have high average levels of circulating antibodies but with high variation between individual fish, as shown in figure 4. Several explanations are possible. Since these samples originate from fish in the field, they might have been exposed to or gone through a subclinical infection of SAV, thereby boosting a primed immune response induced by vaccination. Under such circumstances, some fish will not be infected, and therefore the antibody responses will vary between individuals in the group. It should be added that there was no report of clinical PD but still exposure to live virus can occur. Individual variation in immune responses is often seen for Atlantic salmon, and this also plays into the overall outcome of measured antibody levels.

The DNA vaccine group has a good antibody response, but with some variation in circulating antibody levels. As mentioned above, this could be the product of individual differences in induced responses, possible variations in injection site and volume, and differences in muscular mass and general health, plus variation in size between fish. The titration curve showed an expected decline by dilutions and underscore the level of circulating antibodies present by volume of plasma collected from the fish, however, the reduction over the dilution series is less than for the other vaccines. This is an interesting observation and might reflect that fish immunized with the DNA vaccine raise antibodies with higher affinity; relatively low reduction in OD values with increasing dilution could possibly be explained by higher proportion of antibodies with higher antigen affinity.

The AlphaJect Micro 1 PD group has a lower antibody response than the DNA vaccine group, but with less individual variation. This may indicate that an adjuvanted vaccine, that will induce an inflammation in the abdominal cavity after intraperitoneal injection, induces a

response where the response is more homogenous (than for a DNA vaccine) and thus with lesser individual variation. Factors that influence immune responses in fish are antigen dose [97, 98], use of adjuvants [99, 100], modes of delivery (immersion versus injection) [101], and antigen preparation/vaccine preparations amongst others [102]. The mode of delivery, vaccine preparation and the use of adjuvants differ between the DNA and AlphaJect Micro1 PD (AJ) vaccines. The AJ vaccine is an IWVV where the antigen has been inactivated by use of formalin, which can impact on immunogenicity, *i.e.,* ability or potential to induce immune responses. IWVV will for the main part induce a humoral immune response [103]. The DNA vaccine represents an *in vivo* "production" of the virus antigens, primarily expressed in the muscle at the injection site [104], here the structural proteins, which will be presented to the immune system in a native form, very different from a chemically modified antigen. Immune responses are more diverse and will include both humoral and cellular immunity [105]. This might partly explain the differences in antibody responses between the AJ and DNA groups.

The Comb group (combination of Clynav and AlphaJect Micro 1 PD) has low levels of circulating antibodies, at all dilutions, but with some variation between fish. The antibody levels are skewed towards OD values close to zero (as seen in fig. 7 and 8), i.e. 35-45% of the fish fall in this category (less than 0.2 OD_{450}). The two vaccines were combined from a rational approach that a combination of a DNA and oil-adjuvanted IWV vaccine would strengthen the antibody responses, but the combination has resulted in a negative interaction with lower antibody levels in the Comb group than in any of the two groups given each of the vaccines separately. The reasons for the observed lower antibody levels in the Comb group are not obvious. There is also a possibility that the immune response is delayed, as shown in the study by Skinner et al., where they looked at the antibody responses when vaccinated with a combination of a DNA vaccine i.m. and a polyvalent oil-adjuvant vaccine i.p. [106], and thus that the sampling of plasma was done too early relative to the time of vaccination.

Another possibility for the low antibody response of the combination could be that the oil-adjuvant AlphaJect Micro 1 PD vaccine induces an inflammatory response in the abdomen. This may draw cells to the cavity at an initial stage of the immune responses because of a great migration of immune cells to the site of inflammation, so that there are less B cells available for antibody production against the pathogen injected both intramuscularly and intraperitoneally.

The Kruskal-Wallis test showed that the difference between the DNA, AJ groups and the Comb group is statistically significant, which underlines what was discussed above. The difference between the DNA group and the AJ group is also statistically significant.

ELISA procedure and sources of error

Sources of error that could have occurred during this procedure are air bubbles when pipetting which can impact on the reading, contamination of the pipettes and poor washing of the 96-well plates. The plasma samples could contain less amount of antibodies because the blood was collected before full immunity had developed. The blood samples were taken at 450-degree days post vaccination, it could be that this was too soon for the optimal amount of antibodies to be generated in the vaccinated fish. We find it reasonable that the lack of time to develop optimal immunity, particularly for the combination vaccinated group (comb), should be considered as a source of error.

There is little indication that there have been errors when pipetting the volumes, because the dilution series follows a descending curve (fig. 4). This points in the direction of specificity, with a diluting effect.

Virus harvest and titration

At six days post inoculation we found close to 100% CPE showing that the SAV-3 strain used was infective and the cells permissive, and the virus could be used for virus titration. As figure 10 shows, the first negative CPE was detected in the well at row F/-6 column 6, but the last positive CPE was detected in the well at row G/-7 and column 1. This could reflect that there have been errors in pipetting, like lack of optimal mixing of the dilution above or incorrect volume pipetted. But experienced lab workers have told us that it is not uncommon that CPE is not achieved in all wells in the same dilution row. And also that there can be CPE in some wells with higher dilutions. It is also a possibility of incorrect reading because of lack of experience.

It could be that the well at row F/-6 column 6 (F6) was positive, but due to the previously discussed errors it could have been falsely registered as negative (as shown in figure 10). Due to the use of the last row with 100% CPE in all the wells, in the calculations of the $TCID_{50}$ by the Kärbers method of virus quantifications, this will give a consequential error. Since the F6 well was registered as negative, we had to use row E/-5 as the last 100% CPE positive row. If well F6 had been registered as positive, row F/-6 would then be the last row with 100% CPE and then be used in the titre calculations. By the use of row E/-5 instead of row F/-6 we would get wrong estimates used in the calculation of virus titre by the Kärbers method. With the use of E/-5 we would get a result of 7.50, but with the use of F/-6 we would get a result of 7.67 at results per mL when 100 μL inoculated. When so calculated with 10^x we will find a result for F/-6 like $4.67*10^7$ and E/-5 like $3.16*10^7$. This gives a margin of error (underestimation) at 1.48x (33%). This would give 1.5x more virus added than estimated (150 $TCID_{50}$ instead of 100 $TCID_{50}$).

At day seven we discussed whether or not the well F6 was negative, because it was no clear CPE detected. The well was looked at again at day ten post virus inoculation, where we then concluded that it was negative.

Virus neutralization test and sources of error

The results of the virus neutralization test showed that the plasma did not contain levels of neutralizing antibodies even at 1:2 dilution that could prevent infection of cells. We expected that there would be at least some neutralization in the wells with the lowest dilution factor (1:2), and especially in the wells with the plasma samples from the AlphaJect Micro1 PD vaccinated group, based on the results from the study by Xu et al. [107] that showed that the inactivated whole virus vaccine (AlphaJect micro1 PD) had a superior immunity to DNA vaccines with regard to neutralization of SAV-3.

There can be multiple explanations for lack of induction of neutralizing antibodies. All the wells were read as positive, but a trained eye could have noted that the dilutions had affected the results, and we also observed that the higher dilution, the more pronounced CPE was observed but the neutralizing ability was insufficient to prevent CPE at any dilution. We were not able to examine the samples at an earlier point than seven days post inoculation. This could have shown if some wells with higher dilutions of plasma (lower amount of neutralizing antibodies) had developed CPE at an earlier stage.

In the process of reading the result of the virus titration, there could have been a misreading as described in the discussion of the virus harvest and titration. This could have resulted in a margin of error (underestimation) at 1.48x, based on the last row with 100% CPE to be included in the calculation of the $TCID_{50}$. This would then give a calculated $TCID_{50}$ that could be 150 instead of 100 and this will impact on the amount of virus added for neutralization. We consider this to be of minor probability. A source of error that we consider

to be of major importance of the outcome is the standard of using 100 $TCID_{50}$ for neutralization when testing fish plasma. We think that a serial dilution of virus should have been included in our study (i.e. 50 and 25 in addition to 100 $TCID_{50}$).

Lack of experience reading and evaluating CPE in the light microscope is also a source of error. We do not consider this to be the main reason for the results obtained because the results of the virus titration were checked at both day seven and day ten post inoculation, and we feel confident that the reading was correct at day ten.

There could be a possibility that some of the virus in the supernatant used were non-infective but still with an ability to bind antibodies, which will use up antibodies with less left to neutralize live virus. We see this as a possibility to impact on the results obtained and we have no information regarding infective to non-infective virus in the preparation used.

Another possible source of error that may have contributed to the outcome of the neutralization test, is that plasma samples from the different vaccinated groups were not stored at optimal conditions prior to or during the period of study. As described in Material and Methods, the blood samples from the different groups of fish were kept on ice after collection, and the blood samples were centrifuged at 4 °C. The plasma samples were then stored at -20-25°C. The samples were taken out and thawed in conjunction with the ELISA and then again stored at -20-25°C. The samples were later thawed and used for the virus neutralization test. There could also have been other circumstances where the plasma samples have been thawed, but this is unknown to us. This could have deteriorated the quality of the antibodies and again impacted on neutralization ability. It is not unlikely that this is one source of error that resulted in the lack of neutralization, but we have no conclusive evidence that this is the cause of the obtained results.

Our ELISA did detect antibodies from the plasma samples, but ELISA is known to be a more sensitive laboratory method than virus neutralization test. So even though the ELISA

did detect antibodies, there is a possibility that the levels of antibodies produced in the fish are not at a sufficient level to neutralize the virus.

The sources of error listed above are the ones we find most likely as a possible explanation to the lack of neutralization, but we cannot exclude other factors. We also considered the likelihood of some sort of contamination of the cell medium, or that the cell medium contained ingredients that were not optimal for cell survival and gave a toxic effect that we could have misread as a cytopathic effect. This is not likely due to the presence of the intact negative controls in the different 96-well plates, and that they did not display any toxic effects.

One factor that could be mentioned, but that is not very likely as a source of error, is that the virus used for the virus neutralization test could have been mutated or that the virus used is incorrectly marked as SAV-3, that could result in the failure of the antibodies, sampled from the vaccinated fish, to neutralize the virus which then gave the positive CPE present in all the wells.

Overall discussion of the study, results in general and the sources of error

The generalizability of this study is present, but not optimal because it is mostly a laboratory study and the target group is farmed Atlantic salmon. This is why we used the MSD (Aquavac PD 7) group as a field control. In this study we do not get a picture of how the different environment effects the vaccine response, due to the use of Atlantic salmon from laboratory aquarium.

We think that this study´s statistical power is good, because we tested 25-30 individuals in each vaccine group, but the more individuals in a study, the more accurate the results are.

The ELISA results conclude that antibodies against SAV are present in the different fish groups. The results are most likely reliable, but not statistically significant when comparing the different groups, but it gives us an opportunity to compare most of the antibody levels of the different vaccine groups. The ELISA results showed that the vaccines induced a significant level of circulating antibodies (in most groups/dilutions). Lack of results from the virus neutralization test do not allow us to make any inference as regards protective potential of induced antibodies.

Every step in this research were done to the best of our capability, with focus on following established protocols and maintain a good standard for the hygiene in the laboratory work, to reduce the risk of contamination and sources of error. At the same time, we are not skilled laboratory workers with much experience using laboratory procedures, so it is more likely that unidentified mistakes can have occurred during our laboratory work. During our work with this study there have been multiple people handling our source material, and this might have increased the risk of human bias in our research.

Conclusion

Due to the lack of results from the virus neutralization test, we can only base our conclusion on the valid ELISA results. We can conclude that the ability to induce circulating antibodies in Atlantic salmon is present for the vaccine Clynav (DNA), AlphaJect Micro 1 PD (AJ), and the MSD vaccine (Aquavac PD 7). The MSD vaccine (Aquavac PD 7) induces a higher level of circulating antibodies in Atlantic salmon compared to the DNA and AJ vaccines, and the combination of the two vaccines. We have no valid results in the virus neutralization test and can therefore not compare the levels of neutralizing antibodies elicited by the two different vaccine types, or when the two vaccines are combined and injected simultaneously. Due to this, we can only conclude in this study that the vaccines induce circulating antibodies, but we have no evidence to conclude that the antibodies produced have an ability to neutralize the virus. This needs further research.

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Sammendrag

Tittel: PD-vaksinering – effekt av ulike vaksiner for induksjon av sirkulerende antistoffer

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I denne fordypningsoppgaven har vi undersøkt ulike PD-vaksiners evne til å indusere sirkulerende og nøytraliserende antistoffer i Atlantisk laks. Vi har forsøkt å sammenligne nivået av sirkulerende og nøytraliserende antistoffer fra fisk vaksinert med DNA-vaksinen Clynav og den helvirusinaktiverte vaksinen AlphaJect Micro 1 PD, og kombinasjonen av disse når injisert ved samme tidspunkt. For å detektere nivå og tilstedeværelse av sirkulerende antistoffer i plasmaprøvene benyttet vi metoden indirekte ELISA. Resultatene viste at DNA-vaksinen hadde høyeste nivå av sirkulerende antistoffer, men spredning i nivå innad i gruppen. AlphaJect vaksinen hadde et lavere nivå av sirkulerende antistoffer, men nivået var jevnere innad i gruppen. Kombinasjonen av disse vaksinene hadde et lavt nivå av sirkulerende antistoffer. Vi brukte også en kontrollgruppe hentet fra felt, vaksinert med den helvirus inaktiverte vaksinen kalt Aquavac PD 7 (MSD), som også fikk påvisning av sirkulerende antistoffer, men nivået i gruppen var mer spredt enn i de øvrige gruppene. Dyrket så CHH1-celler og inokulerte disse med SAV-3, og påviste CPE. Deretter utførte vi en virustitrering for å finne $TCID_{50}$, som så ble brukt for virusnøytralisasjonstest. Virusnøytralisasjonstesten hadde ugyldige resultater, hvor alle brønner som inneholdt plasma fra de ulike vaksinegruppene hadde positivt utslag for CPE. Negativ og positiv kontroll var som forventet. Feilkilder til den mislykkede virusnøytralisasjonstesten kan være blant annet dårlig oppbevaring av plasma som kan ha skadet antistoffene, tilstedeværelse av døde virus i den benyttede supernatanten og feil avlesning av brønnrekke som så ble brukt til kalkulering av

TCID$_{50}$. Basert på utfallet av forsøkene kan vi bare konkludere med at det er tilstedeværelse av sirkulerende antistoffer ved de ulike vaksinene og kombinasjonen av de to, i ulikt nivå med høyest nivå i DNA-vaksinerte individer og lavest nivå i kombinasjonsvaksinerte individer. Vi kan ikke si noe om antistoffene er nøytraliserende basert på vårt mislykkede virusnøytralisasjonsresultat.

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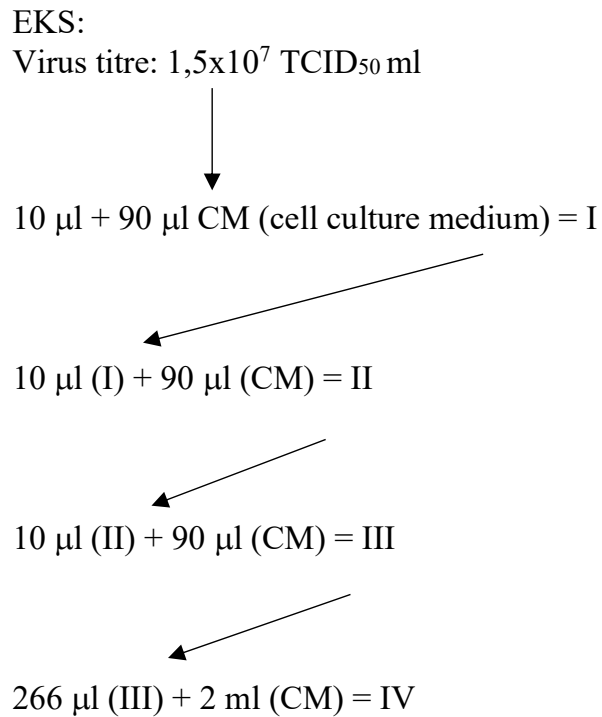
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Appendix

The virus neutralization test

EKS:
Virus titre: $1{,}5 \times 10^7$ TCID$_{50}$ ml

$\downarrow$

$10\ \mu l + 90\ \mu l$ CM (cell culture medium) = I

$10\ \mu l$ (I) + $90\ \mu l$ (CM) = II

$10\ \mu l$ (II) + $90\ \mu l$ (CM) = III

$266\ \mu l$ (III) + 2 ml (CM) = IV

This is an example of how we calculated the diluted virus to 100 TCID$_{50}$ per well.

Our original virus titre was $3{,}16 \times 10^7$ TCID$_{50}$/mL ($10^{7,5}$ TCID$_{50}$/mL).

Raw data ELISA

GENios; Serial number: 12900400526; Firmware: V 4.62 - 07/01 GENios; XFLUOR4 Version: V 4.40					
Date:				4.5.21	
Time:				13:35	
Measurement mode:				Absorbance	
Measurement wavelength:				450	nm
Number of flashes:				3	

Rawdata	plate1				Temperature: 24,4 °C								
◇	1	2	3	4	5	6	7	8	9	10	11	12	
A	0,9703	0,8559	0,6764	0,7465	0,6453	0,5555	0,9574	0,8506	0,7775	0,7604	0,5625	0,6622	
B	0,9973	0,8445	0,7451	0,8145	0,5453	0,5539	0,9651	0,7659	0,7469	0,7679	0,5474	0,6655	
C	0,9464	0,8451	**0,6825**	0,6065	0,5458	**0,6654**	0,6534	0,5043	0,4485	0,9977	0,8701	0,7766	
D	0,9463	0,7344	0,6511	0,6352	0,5462	0,0459	0,6533	0,5483	0,4456	0,8823	0,8123	0,7501	
E	1,5373	1,2973	0,9698	0,8580	0,7975	0,6772	0,7513	0,5465	0,6772	0,8456	0,7494	0,6772	
F	1,5672	1,3280	0,9955	0,8577	0,7525	0,5872	0,7483	0,5457	0,6499	0,8463	0,7512	0,6507	
G	0,9055	0,7754	0,8591	0,8490	0,8470	0,6764	0,6511	0,7045	0,6764	0,0552	1,4920	0,7881	
H	0,9548	0,7517	0,8554	0,9563	0,7527	0,6764	0,6605	0,7510	0,6559	0,0579	1,6744	0,0495	

Rawdata	plate 2												DNA-Vac
◇	1	2	3	4	5	6	7	8	9	10	11	12	Unknown PD-Vac (MSD)
A	1,2517	0,9509	0,8769	1,3502	0,9484	0,8853	0,7692	0,6997	0,6543	0,7575	0,6581	0,5217	Negative control
B	1,2510	0,9474	0,8473	1,3251	0,9455	0,8462	0,7172	0,6901	0,6583	0,7539	0,6518	0,5511	Positive control
C	0,7527	0,6482	0,4261	1,3151	0,8762	0,4589	0,9607	0,6067	0,7212	2,0062	1,3513	0,7562	Blank
D	0,7451	0,6475	0,5487	1,3338	0,8137	0,4186	0,9711	0,5955	0,6913	1,9899	1,2572	0,7743	
E	1,9855	1,6959	1,7013	2,3892	1,7196	1,0349	1,8977	1,3565	1,0912	2,9077	2,5642	1,1036	
F	1,9186	1,8549	1,6259	2,3831	1,7560	1,0796	1,9309	1,2634	1,7982	OVER	2,4533	0,9262	
G	1,3780	1,0016	0,4856	1,2883	0,8557	0,9353	1,3823	1,8778	1,1215	0,0604	1,7833	0,2284	
H	1,5217	1,1451	0,5604	1,5002	0,8308	0,7228	2,0601	1,2027	1,2166	0,0659	1,3766	0,0848	

GENios; Serial number: 12900400526; Firmware: V 4.62 - 07/01 GENios; XFLUOR4 Version: V 4.40		
Date:		5.5.21
Time:		13:43
Measurement mode:		Absorbance
Measurement wavelength:		450 nm
Number of flashes:		3

Rawdata	plate 1				Temperature: 24,5 °C								
◇	1	2	25	4	5	26	7	8	11	10	11	1	
A	2,6768	2,1344	*1,5310*	2,7093	1,9788	*1,1893*	0,4225	0,2927	0,1631	0,0554	0,0517	0,0535	
B	2,5024	2,1582	*1,5199*	2,7001	1,7755	*1,1258*	0,4767	0,3113	0,1791	0,0496	0,0534	0,0486	
C	0,0503	0,0477	0,0561	0,2461	0,1838	0,1175	0,3652	0,3424	0,1520	0,0442	0,0457	0,0455	
D	0,0474	0,0553	0,0596	0,2740	0,2279	0,1227	0,3431	0,3130	0,1560	0,0457	0,0485	0,0457	
E	1,7240	1,8423	*1,3688*	0,0808	0,0561	0,0538	2,8175	2,7664	*2,5035*	0,0533	0,0482	0,0542	
F	2,1176	1,9785	*1,4503*	0,0989	0,0843	0,0523	OVER	OVER	*2,9190*	0,0505	0,0483	0,0468	
G	0,0516	0,0537	0,0490	0,1349	0,0944	0,0715	2,9493	2,8588	*2,8238*	0,0488	0,6110	0,0492	
H	0,0582	0,0606	0,0537	0,1493	0,1045	0,0767	OVER	2,8898	*2,2030*	0,0489	0,6535	0,0475	

Rawdata	plate 2												Combi-Vac
◇	1	2	3	4	5	6	7	8	9	10	11	12	Unknown PD-Vac (MSD)
A	2,7338	2,6913	*2,2579*	0,0504	0,0500	0,0487	0,0504	0,0483	0,0495	2,9810	2,8485	*2,6422*	Negative control
B	2,7871	2,4496	*2,1240*	0,0517	0,0480	0,0499	0,0503	0,0467	0,0450	2,8162	2,1156	*2,0251*	Positive control
C	2,7354	2,3398	*1,9178*	1,6887	1,1769	*0,8200*	0,1597	0,1225	0,0647	0,6124	0,3005	0,2102	Blank
D	2,8094	2,5775	*2,2240*	2,1049	1,5158	*0,7750*	0,2210	0,1456	0,0803	0,4469	0,2674	0,1474	
E	1,8289	1,2045	*0,5976*	0,4659	0,2680	0,1592	0,2685	0,1835	0,1431	1,1998	0,7572	0,3324	
F	1,7026	1,3290	*0,6603*	0,4850	0,2875	0,1594	0,3010	0,1735	0,1084	1,1045	0,7043	0,3012	
G	0,5638	0,3366	0,2059	0,9238	0,8089	0,4459	0,8157	0,6867	0,2837	0,0487	0,9861	0,0462	
H	0,5340	0,3169	0,2019	1,1276	0,7549	0,4759	0,9419	0,6612	0,2171	0,0497	0,9184	0,0471	

GENios; Serial number: 12900400526; Firmware: V 4.62 - 07/01 GENios; XFLUOR4 Version: V 4.40		
Date:		6.5.21
Time:		13:38
Measurement mode:		Absorbance
Measurement wavelength:		450 nm
Number of flashes:		3

Rawdata	Plate 1				Temperature: 24,3 °C									
◇	1	2	3	4	5	6	7	8	9	10	11	12		
A	0,4999	0,3532	0,2476	0,3486	0,2488	0,1533	0,3622	0,2506	0,1813	0,4329	0,2544	0,1727		
B	0,4635	0,3526	0,2486	0,3523	0,2516	0,1661	0,3561	0,2586	0,1771	0,4085	0,2510	0,1828		
C	0,3959	0,3521	0,1493	0,7539	0,5539	0,4701	1,2868	0,8551	0,4303	0,4562	0,2618	0,1982		
D	0,4453	0,3542	0,1494	0,8530	0,6538	0,4497	1,2026	0,7764	0,5047	0,4354	0,2509	0,1703		
E	0,3542	0,2633	0,1508	0,6523	0.3451	0,1669	0,7561	0,3596	0,1995	0,6341	0,421	0,2812		
F	0,3649	0,2498	0,1706	0,5931	0,3821	0,1417	0,6525	0,3518	0,1625	0,6514	0,4521	0,2912		
G	0,4764	0,2879	0,1960	0,6521	0,4498	0,1382	0,4138	0,3395	0,1866	0,0659	1,9233	0,0502		
H	0,5135	0,2778	0,1615	0,6382	0,3968	0,1504	0,4257	0,3611	0,2799	0,0891	1,6623	0,0693		
													Alfa Ject-vac	
													Unknown PD-vac (MSD)	
Rawdata	Plate 2												Negative control	
◇	1	2	3	4	5	6	7	8	9	10	11	12	Positive control	
A	0,4098	0,2743	0,1718	0,3521	0,2898	0,2382	0,5261	0,3177	0,2147	0,4580	0,2576	0,1565	Blank	
B	0,3982	0,1938	0,1488	0,3582	0,2968	0,2504	0,5713	0,3334	0,2169	0,4545	0,2538	0,1502		
C	0,3579	0,2532	0,1210	1,8535	1,2324	0,8701	1,1190	0,6958	0,4985	1,5206	1,0926	0,6557		
D	0,3544	0,2542	0,1525	1,9414	1,3743	0,9537	1,2068	0,6137	0,4869	1,8711	1,1577	0,7327		
E	2,3754	2,0472	1,5741	2,9974	2,6718	1,1246	1,5887	1,0362	0,2147	2,5675	2,1006	1,3551		
F	2,5367	2,0860	1,3987	OVER	OVER	1,3475	1,6375	0,9872	0,5938	2,5244	2,2803	1,6751		
G	1,5899	1,0642	0,6696	0,9341	0,4619	0,3446	0,1903	0,1339	0,0966	0,0545	0,8757	0,0521		
H	1,5857	1,1122	0,7384	0,9241	0,5127	0,3519	0,1936	0,1241	0,1051	0,0528	0,6559	0,0665		

Figures: Used the data hardware Stata SE version 17.

Calculations ELISA

Table Analyzed	Data 1
Data sets analyzed	B, J, M
ANOVA summary	
F	73,81
P value	<0,0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R squared	0,5664
Brown-Forsythe test	
F (DFn, DFd)	3,975 (2, 113)
P value	0,0215
P value summary	*
Are SDs significantly different (P < 0.05)?	Yes
Bartlett's test	
Bartlett's statistic (corrected)	10,21
P value	0,0061
P value summary	**
Are SDs significantly different (P < 0.05)?	Yes

ANOVA table	SS	DF		MS	F (DFn, DFd)	P value
Treatment (between columns)	5,352	2		2,676	F (2, 113) = 73,81	P<0,0001
Residual (within columns)	4,097	113		0,03626		
Total	9,449	115				

Normality of Residuals

Test name	Statistics	P value	Passed normality test (alpha=0,05)?	P value summary
Anderson-Darling (A2*)	5,001	<0,0001	No	****
D'Agostino-Pearson omnibus (K2)	33,85	<0,0001	No	****
Shapiro-Wilk (W)	0,8502	<0,0001	No	****
Kolmogorov-Smirnov (distance)	0,1455	<0,0001	No	****

Data summary	
Number of treatments (columns)	3
Number of values (total)	116

Kärbers metode for kvantifisering av virus (Spearman - Kärber)

"Ny" metode etter orginal beskrivelse

(Beskrivelse fra Virologische Arbeitsmetoden, bind 1, s 39 (på Lindern))

Siste fort. med 100% positive:	5	<=	(Positiv verdi av eksponent i siste fortynning der alle brønner er positive)
Ant. positive brønner f.o.m. 100%:	12	<=	(Antall positive brønner f.o.m. siste rekke der alle er positive)
Fortynningsfaktor:	10		
Antall paralleller:	6		Bruk: - se eksempel =>
Resultat:	6,50		
Resultat pr. ml når 50 µl innokl.:	7,80	<=	
Resultat pr. ml når 100 µl innokl.:	7,50		31622777

Referanser:

Kärber,G. (1931) Beitrag zur kollektiven Behandling pharmakologischer Reihenversuche

Arch.exp.Pathol.Pharmakol. ;162, 480

Spearman,C. (1908)

Brit.J.Psychol. ; 2, 227

Calculation used for row E/-5 (figure 10).

Kärbers metode for kvantifisering av virus (Spearman - Kärber)

"Ny" metode etter orginal beskrivelse

(Beskrivelse fra Virologische Arbeitsmetoden, bind 1, s 39 (på Lindern))

Siste fort. med 100% positive:	6	<=	(Positiv verdi av eksponent i siste fortynning der alle brønner er positive)
Ant. positive brønner f.o.m. 100%:	7	<=	(Antall positive brønner f.o.m. siste rekke der alle er positive)
Fortynningsfaktor:	10		
Antall paralleller:	6		Bruk: - se eksempel =>
Resultat:	6,67		
Resultat pr. ml når 50 µl innokl.:	7,97	<=	
Resultat pr. ml når 100 µl innokl.:	7,67		46415888

Referanser:

Kärber,G. (1931) Beitrag zur kollektiven Behandling pharmakologischer Reihenversuche

Arch.exp.Pathol.Pharmakol. ;162, 480

Spearman,C. (1908)

Brit.J.Psychol. ; 2, 227

Calculation used for row F/-6 (figure 10).