CHAPTER 2.3.6.

INFECTION WITH SALMONID ALPHAVIRUS

1. Scope

Infection with salmonid alphavirus (SAV) means infection with any genotype of the pathogenic agent SAV, of the Genus *Alphavirus* and Family *Togaviridae*.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

SAV is an enveloped, spherical, single-stranded, positive-sense RNA virus, approximately 60–70 nm in diameter, with a genome of ~12 kb. The genome codes for eight proteins: four capsid glycoproteins (E1, E2, E3 and 6K) and four nonstructural proteins (nsP1–4). Glycoprotein E2 is considered to be the site of most neutralising epitopes, while E1 contains more conserved, cross-reactive epitopes (McLoughlin & Graham, 2007). SAV is considered to belong to the genus *Alphavirus* of the family *Togaviridae*. This is based on nucleotide sequence studies of SAV isolates, and is also supported by biological properties of the virus, including cross-infection and neutralisation trials. In addition, four conserved nucleotide sequence elements (CSEs) and a conserved motif (GDD), characteristic of alphaviruses, are present in the SAV genome (McLoughlin & Graham, 2007).

SAV has been divided into six genotypes (SAV1–SAV6) based solely on nucleic acid sequence for the proteins E2 and nsP3 (Fringuelli et al., 2008). The level of antigenic variation among genotypes is considered low as monoclonal antibodies (MAbs) raised against a specific SAV subtype are likely to cross react with other SAV isolates (Graham et al., 2014; Jewhurst et al., 2004).

Infection with SAV may cause pancreas disease (PD) or sleeping disease (SD) in Atlantic salmon (*Salmo salar* L.), common dab (*Limanda limanda*), rainbow trout (*Oncorhynchus mykiss*) (McLoughlin & Graham, 2007) and Arctic charr (*Salvelinus alpinus*) (Lewisch et al., 2018). The disease is a systemic disease characterised microscopically by necrosis and loss of exocrine pancreatic tissue, and heart and skeletal muscle changes.

The genotype groups by susceptible species and environment are presented in Table 2.1.

<table>
<thead>
<tr>
<th>SAV genotype</th>
<th>Freshwater</th>
<th>Sea water</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAV 1</td>
<td>Rainbow trout</td>
<td>Atlantic salmon</td>
</tr>
<tr>
<td>SAV 2</td>
<td>Rainbow trout</td>
<td>Atlantic salmon</td>
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<tr>
<td></td>
<td>Atlantic salmon</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Arctic charr</td>
<td></td>
</tr>
<tr>
<td>SAV 2</td>
<td>Atlantic salmon</td>
<td></td>
</tr>
<tr>
<td>SAV 3</td>
<td>Rainbow trout</td>
<td>Atlantic salmon</td>
</tr>
<tr>
<td></td>
<td>Atlantic salmon</td>
<td></td>
</tr>
<tr>
<td>SAV 4</td>
<td>Atlantic salmon</td>
<td></td>
</tr>
<tr>
<td>SAV 5</td>
<td>Atlantic salmon</td>
<td>Common dab</td>
</tr>
<tr>
<td>SAV 6</td>
<td>Atlantic salmon</td>
<td></td>
</tr>
</tbody>
</table>
2.1.2. Survival outside the host

Laboratory tests suggest that SAV would survive for extended periods in the aquatic environment. In these tests, virus survival was inversely related to temperature. In the presence of organic matter, marked longer survival times were observed in sea water compared with fresh water (Graham et al., 2007c). SAV has been detected in fat leaking from dead fish, indicating that this may be a route for transmission. Fat droplets may accumulate at the sea water surface, contributing to long distance spread (Stene et al., 2015).

The half-life of SAV in serum has been found to be inversely related to temperature, emphasising the need for rapid shipment of samples at 4°C to laboratories for virus isolation. For long-term conservation of SAV-positive samples and cultured virus, storage at –80°C is recommended (Graham et al., 2007c).

2.1.3. Stability of the agent (effective inactivation methods)

SAV is rapidly inactivated in the presence of high levels of organic matter at 60°C, at pH 7.2, and at pH 4 and pH 12 at 4°C, suggesting that composting, ensiling and alkaline hydrolysis would all be effective at inactivating virus in fish waste (Graham et al., 2007a).

2.1.4. Life cycle

Probable infection routes are through the gills or via the intestine. In the acute stages of the disease, large amounts of SAV can be detected and live virus can be isolated from the heart, kidney, blood and several other organs, but the actual target cells for the virus has not yet been identified.

Viraemia precedes both the onset of histological changes and clinical signs (McLoughlin & Graham, 2007). The route of shedding may be through natural excretions/secretions, supported by the detection of SAV by reverse-transcriptase polymerase chain reaction (RT-PCR) in the faeces and mucus of experimentally infected Atlantic salmon. These matrices may therefore play a role in the horizontal transmission of SAV through water (Graham et al., 2012). Virus has been detected in water 4–13 days after infection, indicating that virus shedding coincides with the viraemic stage (Andersen et al., 2010). An incubation period of 7–10 days at sea water temperatures of 12–15°C has been estimated based on analysis of antibody production in intraperitoneally infected fish and cohabitants in an experimental trial (McLoughlin & Graham, 2007). Several studies have shown that SAV RNA can be detected in fish for an extended period post-infection (Jansen et al., 2010a; McLoughlin & Graham, 2007). Subclinical infection has been reported, suggesting that the severity of an outbreak may be influenced by several environmental factors (McLoughlin & Graham, 2007), and seasonal increases in water temperature may trigger disease outbreaks (Stene et al., 2014).

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with SAV according to Chapter 1.5. of the Aquatic Animal Health Code (Aquatic Code) include: Arctic charr (Salvelinus alpinus), Atlantic salmon (Salmo salar), common dab (Limanda limanda) and rainbow trout (Oncorhyncus mykiss).

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence for susceptibility according to Chapter 1.5. of the Aquatic Code include: long rough dab (Hippoglossoides platessoides), plaice (Pleuronectes platessa) and Ballan wrasse (Labrus bergylta).

In addition, pathogen-specific positive PCR results have been reported in the following species, but an active infection has not been demonstrated: Argentine hake (Merluccius hubbsi), brown trout (Salmo trutta), cod (Gadus morhua), European flounder (Platichthys flesus), haddock (Melanogrammus aeglefinus), herring (Glupea harengus), Norway pout (Trisopterus esmarkii), saithe (Pollachius virens), sculpin sp. (Myxoceophalus octodecemspinosus) and whiting (Merlangius merlangus).

2.2.3. Susceptible stages of the host

All life stages should be considered as susceptible to infection with SAV.

Farmed rainbow trout in fresh water are affected at all stages of production (Kerbart Boscher et al., 2006). Experience from Norway show that farmed rainbow trout and Atlantic salmon are susceptible at all stages in
sea water, probably reflecting a sea water reservoir of SAV. Experimental infection by injection indicates susceptibility of Atlantic salmon parr in fresh water (McVicar, 1990).

### 2.2.4. Species or subpopulation predilection (probability of detection)

There is no known species or subpopulation predilection.

### 2.2.5. Target organs and infected tissue

Infection with SAV is a systemic disease with an early viraemic phase. After infection, SAV has been detected in all organs that have been examined: brain, gill, pseudobranch, heart, pancreas, kidney and skeletal muscle (Andersen et al., 2007; McLoughlin & Graham, 2007) as well as in mucus and faeces (Graham et al., 2012).

### 2.2.6. Persistent infection with lifelong carriers

SAV has been detected in surviving fish 6 months after experimental infection (Andersen et al., 2007). At the farm level, an infected population will harbour SAV until slaughter (Jansen et al., 2010a; Jansen et al., 2010b). On an individual level, however, lifelong persistent infection has not been documented.

### 2.2.7. Vectors

SAV has been detected by RT-PCR in salmon lice (Lepeophtheirus salmonis) collected during acute disease outbreaks in Atlantic salmon, but transfer to susceptible fish species has not been studied (Petterson et al., 2009). Vectors are not needed for transmission of SAV.

### 2.2.8. Suspected aquatic animal carriers

In surveys of wild marine fish, SAV RNA has been detected in the flatfish species common dab (Limanda limanda), long rough dab (Hippoglossoides platessoides) and plaice (Pleuronectes platessa) (McCleary et al., 2014; Snow et al., 2010). The importance of wild marine or fresh water species as carriers needs to be determined.

### 2.3. Disease pattern

#### 2.3.1. Transmission mechanisms

Transmission of SAV occurs horizontally. This is supported by phylogenetic studies, successful transmission among fish in cohabitant studies, proven transmission between farming sites, studies on survival of SAV in sea water and the spread via water currents (Graham et al., 2007c; Graham et al., 2011; Jansen et al., 2010a; Kristoffersen et al., 2009; Viljugrein et al., 2009).

Long-distance transmission and thus introduction of SAV in a previously uninfected area is most likely assigned to movement of infected live fish (Kristoffersen et al., 2009; Rodger & Mitchell, 2007). Once SAV has been introduced into an area, farm proximity and water currents are factors involved in local transmission (Aldrin et al., 2010; Kristoffersen et al., 2009; Viljugrein et al., 2009). Risk factors for outbreaks on a farming site include a previous history of infection with SAV, high feeding rate, high sea lice burden, the use of autumn smolts and previous outbreak of infectious pancreas necrosis (IPN) (Bang Jensen et al., 2012; Kristoffersen et al., 2009; Rodger & Mitchell, 2007).

Vertical transmission of SAV has been suggested (Bratland & Nylund, 2009), but the evidence is not convincing (Kongtorp et al., 2010; McLoughlin & Graham, 2007). The Norwegian Scientific Committee for Food Safety (2010)1 carried out a risk assessment and concluded that the risk of vertical transmission of SAV is negligible.

#### 2.3.2. Prevalence

The prevalence of infection with SAV may vary. During disease outbreaks, the prevalence is usually high; prevalences of 70–100% have been reported in Atlantic salmon farming sites (Graham et al., 2010). If

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1 The Norwegian Scientific Committee for Food Safety (Vitenskapskomiteen for Mattrygghet) (2010). Risikovurdering - stamfiskovervåking og vertikal smitteoverfering. 01. 1-44. Available at: https://vkm.no/download/18.a665c1015c855db0a71500464589864/Vurdering%20av%20sannsynlighet%20for%20og%20risiko%20ved%20vertikal%20overf%C3%B8ring%20av%20smitte.pdf
moribund or thin fish or runts are sampled, the probability of detecting SAV is higher than if randomly selected, apparently healthy fish are sampled (Jansen et al., 2010b). Prevalence estimates will also vary with the diagnostic method used.

Prevalence in wild fish is largely unknown. SAV RNA has been detected in some flatfish species in sea water in Scotland (Snow et al., 2010). A serological survey of wild salmonids in fresh water river systems in Northern Ireland did not detect virus neutralisation antibodies against SAV in any of 188 sera tested, whereas the majority of sera from farmed salmon in sea water in the same area tested positive (Graham et al., 2003).

### 2.3.3. Geographical distribution

Infection with SAV is known to be present in farmed salmonid fish in Croatia, France, Germany, Ireland, Italy, Norway, Poland, Spain, Switzerland and the United Kingdom (England, Scotland and Northern Ireland).

### 2.3.4. Mortality and morbidity

Mortality rates due to infection with SAV may vary with genotype, season, year, use of biosecurity measures and species of fish (Bang Jensen et al., 2012; Graham et al., 2011; Rodger & Mitchell, 2007; Stormoen et al., 2013). The cumulative mortality at the farm level ranges from negligible to over 50% in severe cases (Bang Jensen et al., 2012; Graham et al., 2003; Rodger & Mitchell, 2007; Ruane et al., 2008; Stene et al., 2014).

Duration of disease outbreaks, defined as the period with increased mortality, varies from 1 to 32 weeks (Jansen et al., 2010a; Jansen et al., 2014; Ruane et al., 2008).

### 2.3.5. Environmental factors

Clinical outbreaks and mortality are influenced by water temperature and season (McLoughlin & Graham, 2007; Rodger & Mitchell, 2007; Stene et al., 2014; Stormoen et al., 2013). Stressing the fish by movement, crowding or treatment may initiate disease outbreaks on infected farms.

### 2.4. Control and prevention

#### 2.4.1. Vaccination

DNA-based and virus-inactivated vaccines against SAV are both commercially available.

#### 2.4.2. Chemotherapy

No chemotherapy is available.

#### 2.4.3. Immunostimulation

No immunostimulation is available.

#### 2.4.4. Resistance breeding

Differences in susceptibility among different family groups of Atlantic salmon have been observed in challenge experiments and in the field, indicating the potential for resistance breeding. Both in Ireland and Norway, efforts are being made to breed fish that are more resistant to infection with SAV (McLoughlin & Graham, 2007).

#### 2.4.5. Restocking with resistant species

Some important culture species, including Nile tilapia, milk fish and Chinese carp, have been shown to be resistant to EUS and could be cultured in endemic areas. Introducing resistant indigenous fish species is recommended.

#### 2.4.6. Blocking agents

Not relevant.
2.4.7. Disinfection of eggs and larvae

Disinfection procedures were evaluated in fertilised ova from SAV genotype 3 positive broodstock (Kongtorp et al., 2010). Nevertheless, further investigation is needed.

2.4.8. General husbandry practices

To avoid infection with SAV, general good hygiene practices should be applied: use of appropriate sites for farming, segregation of generations, stocking with good quality fish, removal of dead fish, regular cleaning of tanks and pens, controlling parasites and other pathogens as well as careful handling of fish. Once a site has been infected, mortality may be reduced by imposing a general stop on handling of the fish as well as a general stop on feeding the fish.

3. Sampling

3.1. Selection of individual specimens

All production units (ponds, tanks, net-cages, etc.) should be inspected for the presence of dead, weak or abnormally behaving fish. Extremely weak («sleeping») fish may be found at the bottom of a tank or in the net-cages. If the number of clinically diseased fish is low, samples from long, thin fish («runts») may be added (Jansen et al., 2010b).

3.2. Preservation of samples for submission

<table>
<thead>
<tr>
<th>Method</th>
<th>Preservative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histology and immunohistochemistry:</td>
<td>Fixation in neutral phosphate-buffered 10% formalin</td>
</tr>
<tr>
<td>Molecular biology (RT-PCR and sequencing):</td>
<td>Appropriate medium for preservation of RNA</td>
</tr>
<tr>
<td>Cell culture:</td>
<td>Virus transport medium</td>
</tr>
<tr>
<td>Serology:</td>
<td>Blood plasma or serum</td>
</tr>
</tbody>
</table>

3.3. Pooling of samples

Pooling of samples may be acceptable, however, the impact on sensitivity and design prevalence must be considered.

3.4. Best organs or tissues

Heart and mid-kidney are the recommended organs for detection of SAV either by molecular biological methods or by cell culture. During the course of the disease, the heart usually contains more SAV than other tissues and should always be sampled. After disease outbreaks, gills and heart (Graham et al., 2010) and pools of heart and mid-kidney (Jansen et al., 2010a; Jansen et al., 2010b) remained RT-PCR positive for months after initial detection.

During the initial viraemic phase, serum samples are also suitable for detection of SAV either by molecular biological methods or by cell culture. Serum sampling may therefore be used for early warning screening tests (Graham et al., 2010). From approximately 3 weeks after SAV infection, blood serum or plasma is suitable for a virus neutralisation test that identifies neutralising antibodies against SAV in fish exposed to SAV (Graham et al., 2003).

Tissues for histological examinations should include gill, heart, pyloric caeca with attached pancreatic tissue, liver, kidney, spleen and skeletal muscle containing both red (aerobe) and white (anaerobe) muscle. Skin with associated skeletal muscle sample should be taken at the lateral line level and deep enough to include both red and white muscle.
4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

A sudden drop in appetite may be observed 1–2 weeks before the detection of elevated mortality. Clinically diseased fish may be observed swimming slowly at the water surface. In some cases, extremely weak («sleeping») fish can be found at the bottom of tanks or in net-cages. An increased number of faecal casts may also be observed. However, it is important to note that clinical signs are not pathognomonic. Careful investigation of any dead, moribund or abnormally behaving fish is necessary to determine involvement of SAV and rule out other pathogenic agents.

Initially, nutritional status is usually normal, but in the months after an outbreak or in the later stages of disease, long slender fish («runts») with poor body condition are typically observed. The presentation of long, slender fish can be caused by factors other than SAV.

4.2. Clinical methods

4.2.1. Gross pathology

Yellow mucoid gut contents are a usual post-mortem finding, as is typically seen in fish that are not eating. Occasionally signs of circulatory disturbances, such as petechial haemorrhages, small ascites or reddening of the pancreatic region between the pyloric caeca, may be seen. Some diseased fish may show pale hearts or heart ruptures. It is important to note that post-mortem findings are not pathognomonic.

4.2.2. Clinical chemistry

Not documented for diagnostic use

4.2.3. Microscopic pathology

The changes most commonly found in clinically diseased fish are severe loss of exocrine pancreatic tissue, cardiomyocytic necrosis and inflammation, red (aerobe) skeletal muscle inflammation and white (anaerobe) skeletal muscle degeneration or inflammation. A less frequent but supporting finding is the detection of cells with many cytoplasmic eosinophilic granules along kidney sinusoids.

As the disease progresses, the development of these changes is not simultaneous in all organs: In a very short, early phase, the only lesion present can be necrosis of exocrine pancreatic tissue and a variable inflammatory reaction in the peripancreatic fat. Shortly thereafter, heart muscle cell degeneration and necrosis develop before the inflammation response in the heart becomes more pronounced. The pancreatic necrotic debris will seemingly disappear and the typical picture of severe loss of exocrine pancreatic tissue will soon appear simultaneously with the increasing inflammation in the heart. Somewhat later, skeletal muscle degeneration, inflammation and fibrosis develop. In a proportion of fish, severe fibrosis of the peri-acinar tissue may occur, and in this case the pancreas does not recover (runts) (Christie et al., 2007; Kerbart Boscher et al., 2006; McLoughlin & Graham, 2007; Taksdal et al., 2007).

4.2.4. Wet mounts

Not relevant.

4.2.5. Smears

Not relevant.

4.2.6. Fixed sections, immunohistochemistry

Immunohistochemical testing (Taksdal et al., 2007) is only recommended for samples from fish with acute necrosis of exocrine pancreatic tissue.
4.2.6.1. Preparation of tissue sections

The tissues are fixed in neutral phosphate-buffered 10% formalin for at least 1 day, dehydrated in graded ethanol, cleared in xylene and embedded in paraffin, according to standard protocols. Approximately 3 µm thick sections (for immunohistochemistry sampled on poly-L-lysine-coated slides) are heated at 56–58°C (maximum 60°C) for 20 minutes, dewaxed in xylene, rehydrated through graded ethanol, and stained with haematoxylin and eosin for histopathology and immunohistochemistry as described below.

4.2.6.2. Staining procedure for immunohistochemistry

All incubations are carried out at room temperature and all washing steps are done with Tris-buffered saline (TBS).

i) Nonspecific antibody binding sites are first blocked in 5% bovine serum albumin (BSA) in TBS for 20 minutes. The solution is then poured off without washing.

ii) Sections are incubated with primary antibody (monoclonal mouse antibody 4H1 against E1 SAV glycoprotein [Todd et al., 2001]), diluted 1/3000 in 2.5% BSA in TBS and then incubated overnight, followed by two wash out baths lasting a minimum of 5 minutes.

iii) Sections are incubated with secondary antibody (biotinylated rabbit anti-mouse Ig) diluted 1/300 for 30 minutes, followed by wash out baths as in step ii above.

iv) Sections are incubated with streptavidin with alkaline phosphatase 1/500 for 30 minutes followed by wash out baths as in step ii above.

v) For detection of bound antibodies, sections are incubated with Fast Red 2 (1 mg ml⁻¹) and Naphthol AS-MX phosphate (0.2 mg ml⁻¹) with 1 mM Levamisole in 0.1 M TBS (pH 8.2) and allowed to develop for 20 minutes followed by one wash in tap water before counterstaining with Mayer's haematoxylin and mounting in aqueous mounting medium.

SAV-positive and SAV-negative tissue sections are included as controls in every setup (Taksdal et al., 2007).

4.2.7. Electron microscopy/cytopathology

Not relevant for diagnostic use.

4.2.8. Differential diagnoses

4.2.8.1. Differential diagnoses relevant for microscopic pathology (Section Microscopic pathology)

Tissues that are changed by infection with SAV are also changed by heart and skeletal muscle inflammation (HSMI), cardiomyopathy syndrome (CMS) and IPN. However, if all the main organs are examined by histopathology, the pattern of affected organs will usually appear different as illustrated in Table 4.1.

<table>
<thead>
<tr>
<th></th>
<th>Infection with SAV</th>
<th>HSMI</th>
<th>CMS</th>
<th>IPN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart*</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Pancreas</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Heart changes in CMS affects mainly the inner spongy layer of the ventricle and the atrium, whereas in Infection with SAV and HSMI, the compact layer of the ventricle is more severely affected. Although these three diseases induce epicarditis, HSMI causes the most severely inflamed epicardium.

In a very short, early acute stage of infection, when only necrosis of exocrine pancreas has developed, infection with SAV might be mistaken for IPN caused by infection with IPN virus (IPNV). In such cases, virological examination will clarify the causal agent.

Reference to specific commercial products as examples does not imply their endorsement by the OIE. This applies to all commercial products referred to in this Aquatic Manual.
Virological and serological examinations combined with histopathological examination of 5–10 clinically diseased fish will usually clarify the situation. HSMI and CMS have only been detected in Atlantic salmon.

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

4.3.1.1. Agent isolation and identification

4.3.1.1.1. Cell culture

Isolation of field isolates of SAV in cell culture may be challenging (Christie et al., 1998; Graham et al., 2007c; Petterson et al., 2013). CHSE-214 are commonly used for primary SAV isolation, but susceptible cell lines such as BF-2, FHM, SHK-1, EPC, CHH-1 or others, may be used. Variation in cell line susceptibility among different SAV field isolates has been reported (Graham et al., 2008; Herath et al., 2009), and it is therefore recommended that several cell lines are tested for initial cell culture isolation of SAV in a new laboratory or for a new virus strain.

The CHSE-214 cells are grown at 20°C in Eagle’s minimal essential medium (EMEM) with non-essential amino acids and 0.01 M HEPES (N-2-hydroxyethyl-piperazin e-N-2-ethanesulfonic acid) buffer, or Leibovitz’s L-15 cell culture medium, both supplemented with fetal bovine serum (FBS) (5% or 10%) and L-glutamine (4 mM)

For virus isolation, cells are grown in tissue culture flasks or multi-well cell culture plates. SAV-positive controls may be inoculated in parallel with the tissue samples as a test for cell susceptibility to SAV. When positive controls are included, measures must be taken to avoid contamination.

i) Inoculation of cell monolayers

Prepare a 2% suspension of tissue homogenate or a 10% suspension of serum using L-15 medium or EMEM without serum or other medium with documented suitability. Remove growth medium from actively growing monolayers (1- to 2-day-old cultures or cultures of 70–80% confluency) grown in tissue culture flasks or multi-well cell culture plates (see above). Inoculate monolayers with a low volume of the 2% tissue homogenate or 10% serum dilution (for 25 cm² flasks: 1.5 ml). Adjust volume to the respective surface area in use. Allow 2–3 hours’ incubation at 15°C followed by removal of the inoculum, and addition of fresh L-15 or EMEM medium supplemented with 2–5% fetal bovine serum (for 25 cm² flasks: 5 ml).

When fish samples come from production sites where IPNV is regarded as endemic, the tissue homogenate supernatants should be incubated (for a minimum of 1 hour at 15°C) with a pool of antisera to the indigenous serotypes of IPNV prior to inoculation.

ii) Monitoring incubation

Inoculated cell cultures (kept at 15°C) are examined at regular intervals (at least every 7 days) for the occurrence of cytopathic effect (CPE). Typical CPE due to SAV appears as plaques of pyknotic, vacuolated cells. However, Norwegian SAV field isolates (both SAV3 and marine SAV2) usually do not produce CPE in low passages, and this is also reported for other SAV genotypes (Graham et al., 2008; Petterson et al., 2013). If no CPE has developed after 14 days, subculture to fresh cell cultures.

iii) Subcultivation procedure

14 days (or earlier when obvious CPE appears) after inoculation, the cultures are freeze–thawed at –80°C (the procedure can be repeated 1–2 times) to release virus from the infected cells.

Following centrifugation at 3000 g for 5 minutes, the supernatants are inoculated into fresh cell cultures as described for the primary inoculation: remove growth medium, inoculate monolayers with a small volume of diluted supernatant (1/5 and higher dilutions) for 2–3 hours before addition of fresh medium.

Inoculated cell cultures are incubated for at least 14 days and examined at regular intervals, as described for the primary inoculation. At the end of the incubation period, or earlier if obvious CPE appears, the medium is collected for virus identification, as described below. Cell cultures should
always be examined for the presence of SAV by immunofluorescence (indirect fluorescent antibody test [IFAT]), as virus replication may occur without development of apparent CPE.

iv) Antibody-based verification of SAV growth in cell culture

All incubations below are carried out at room temperature unless otherwise stated.

a) Prepare monolayers of cells in appropriate tissue culture plates (e.g. 96-well plates), or on cover-slips, depending on the type of microscope available (an inverted microscope equipped with UV light is necessary for monolayers grown on tissue culture plates). The necessary monolayers for negative and positive controls must be included.

b) Inoculate the monolayers with the virus suspensions to be identified in tenfold dilutions, two monolayers for each dilution. Add positive virus control in dilutions known to give a good staining reaction. Incubate inoculated cell cultures at 15°C for 9–11 days.

c) Fix in 80% acetone for 20 minutes after removing cell culture medium and rinsing once with 80% acetone. Remove the fixative and air dry for 1 hour. If necessary, the fixed cell cultures may be stored dry for 14 days at 4°C until staining.

d) Incubate the cell monolayers with anti-SAV MAb in an appropriate dilution in phosphate-buffered saline (PBS) for 1 hour and rinse three times with PBS with 0.05% Tween 20.

e) Incubate with fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin for 1 hour (or if the primary Ab is polyclonal from rabbits, use FITC-conjugated antibody against rabbit immunoglobulin), according to the instructions of the supplier. To increase the sensitivity of the test, FITC-conjugated anti-mouse Ig may be replaced with biotin-labelled anti-mouse Ig and FITC-labelled streptavidin with rinsing as in step d in between the steps. The nuclei can be stained with propidium iodide (100 µg ml⁻¹ in sterile distilled water). Add PBS (without Tween 20) and examine under UV light. To avoid fading, the stained plates should be kept in the dark until examination. For long periods of storage (more than 2–3 weeks) a solution of 1,4-diazabicyclooctane (DABCO 2.5% in PBS, pH 8.2) or similar reagent may be added as an anti-fade solution.

4.3.1.1.2. Reverse-transcription polymerase chain reaction (RT-PCR), real-time RT-PCR, and genotyping by sequencing

The primers described below for real-time RT-PCR and RT-PCR with sequencing will detect all known genotypes of SAV.

RT-PCR may be used for detection of SAV from total RNA (or total nucleic acids) extracted from recommended organs or tissues (see Section 3.4). Real-time RT-PCR for the detection of SAV is recommended as it increases the specificity and also the sensitivity of the test.

For genotyping, RT-PCR with subsequent sequencing of fragments from the E2 gene is recommended.

The primers and probe sequences for real-time RT-PCR from the nsP1 gene, as well as primers for genotyping, are listed in Table 4.2. The E2-primers may also be used for conventional RT-PCR detection of SAV, if necessary. A variety of kits designed for RNA extraction/RT-PCR and qPCR machines can be used. The PCR programme depends on the kit and real-time PCR equipment used in the laboratory. The conditions for performing the real-time RT-PCR in the OIE Reference Laboratory is as follows: 50°C for 10 minutes, 95°C for 3 minutes, and 40 cycles of (95°C for 10 seconds, 60°C for 20 seconds). For the conventional RT-PCRs (sequencing), the following programme is used: 50°C for 30 minutes, 95°C for 15 minutes, and 45 cycles of (94°C for 60 seconds, 55°C for 45 seconds, 72°C for 60 seconds).
### 4.3.2. Serological methods

#### 4.3.2.1. Immunoperoxidase-based serum neutralisation assay (Graham et al., 2003)

Experimental studies have shown that neutralising antibodies can first be detected 10–16 days post-infection (Graham et al., 2003), and serum neutralisation (SN) assays can be used as a diagnostic tool for the detection of SAV antibodies. SN assays are based on the presence or absence of detectable virus growth in cultured cells following incubation with serum that may contain neutralising antibodies. In addition, the assay allows detection of virus in serum or plasma, if present.

CHSE-214 cells are grown as described in Section 4.3.1.1. Cell culture. A suspension of trypsinised cells, diluted 1/3 in growth medium (10% FBS) is prepared for the SN assay.

1. 1/20 and 1/40 dilutions of each test serum are prepared in maintenance medium (2% FBS), and transferred to two duplicate wells (15 µl per well) on a flat-bottomed tissue culture grade microtitre plate. An equal volume of virus (100 TCID<sub>50</sub> [median tissue culture infective dose]) is added and the plate is incubated for 2 hours at room temperature.

2. 70 µl of maintenance medium, and 50 µl of the CHSE-214 cell suspension is added to each well, and the plates are incubated for 3 days at 15°C.

3. The cell monolayer is then fixed and stained as described in Section 4.3.1.1. step iv Antibody-based verification of SAV growth in cell culture, or using the following procedure: monolayers of CHSE-214 cells are fixed for 30 minutes at room temperature in 10% neutral buffered formalin. Following two washes with 0.01 M PBS, a MAb against SAV is added to the monolayers in an appropriate dilution. Bound MAb is visualised using a labelled streptavidin-biotin system according to the manufacturer's instructions.

4. SN titres (ND<sub>50</sub>) are then calculated according to the method of Karber (1931), with titres >1:20 being considered positive. Both serum controls (without virus added) and a virus control (without serum added) must always be included in the assay, to ensure valid results.

### 5. Rating of tests against purpose of use

As an example, the methods currently available for targeted surveillance and diagnosis of infection with SAV are listed in Table 5.1. The designations used in the Table indicate: a = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; b = the method is a standard method with good diagnostic sensitivity and specificity; c = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and d = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category a or b have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

#### Table 4.2. Primers and probe sequences for RT-PCR and real-time RT-PCR

<table>
<thead>
<tr>
<th>Primer and probe sequences</th>
<th>Genomic segment</th>
<th>Product size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>QnsP1F: 5'-CCG-GCC-CTG-AAC-CAG-TT-3'</td>
<td>QnsP1</td>
<td>107 nt</td>
<td>1</td>
</tr>
<tr>
<td>QnsP1R: 5'-GTA-GCC-AAG-TGG-GAG-AAA-GCT-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QnsP1probe: 5'FAM-CTG-GCC-ACC-ACT-TCG-A-MGB3' (Taqman®probe)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2F: 5'-CCG-TTG-CGG-CCA-CAC-TGG-ATG-3'</td>
<td>E2</td>
<td>516 nt</td>
<td>2</td>
</tr>
<tr>
<td>E2R: 5'-CCT-CAT-AGG-TGA-TCG-ACG-GCA-G-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 = Hodneland & Endresen, 2006. 2 = Fringuelli et al., 2008.

#### Table 5.1. Methods for targeted surveillance and diagnosis

<table>
<thead>
<tr>
<th>Method</th>
<th>Targeted surveillance</th>
<th>Presumptive diagnosis</th>
<th>Confirmatory diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gross signs</td>
<td>Fry</td>
<td>Juveniles</td>
<td>Adults</td>
</tr>
<tr>
<td>d</td>
<td>d</td>
<td>d</td>
<td>c</td>
</tr>
</tbody>
</table>
6. Test(s) recommended for targeted surveillance to declare freedom from infection with SAV

The recommended test to be used in surveillance of susceptible fish populations for declaration of freedom from SAV is RT-PCR as described in Section 4.3.1.1.2 in this chapter.

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

A suspected case of infection with SAV is defined as:

i) Clinical signs consistent with infection with SAV (Section 4.1.1) or

ii) Gross and microscopic pathology consistent with the disease (Sections 4.2.1 and 4.2.3) or

iii) Detection of antibodies against SAV (Section 4.3.2.1) or detection of SAV (Section 4.3.1.1.) or

iv) If epidemiological information of infectious contact with suspected or confirmed case(s) appears.

7.2. Definition of confirmed case

Evidence for the presence of SAV from two independent laboratory tests as microscopic pathology (Section 4.2.3), cell culture (Section 4.3.1.1.1), RT-PCR (Section 4.3.1.1.2) or serology (Section 4.3.2).

8. References


Chapter 2.3.6. - Infection with salmonid alphavirus


**NB:** There is an OIE Reference Laboratory for infection with salmonid alphavirus (see Table at the end of this or consult the OIE web site for the most up-to-date list: [http://www.oie.int/en/scientific-expertise/reference-laboratories/list-of-laboratories/](http://www.oie.int/en/scientific-expertise/reference-laboratories/list-of-laboratories/)). Please contact the OIE Reference Laboratory for any further information on infection with salmonid alphavirus.

**NB:** FIRST ADOPTED IN 2014; MOST RECENT UPDATES ADOPTED IN 2019.