CHAPTER 2.3.3.

INFECTION WITH *GYRODACTYLUS SALARIS*

1. Scope

Infection with *Gyrodactylus salaris* means infection with the pathogenic agent *Gyrodactylus salaris*, a viviparous ectoparasite of the Family *Gyrodactylidae* and Class *Monogenea*.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

Several strains of *G. salaris* have been identified on the basis of genotyping with the mitochondrial cytochrome oxidase 1 (CO1) marker (Hansen *et al*., 2003; 2007b; Meinilä *et al*., 2002; 2004; Mieszkowska *et al*., 2018). Although there does not seem to be an unambiguous correspondence between parasite strains as identified by CO1 and pathogenicity (Hansen *et al*., 2007a), all strains recovered from Atlantic salmon (*Salmo salar*) that have been studied in laboratory experiments, so far, are highly pathogenic to strains of Atlantic salmon. Strains non-pathogenic to Atlantic salmon have been recovered from non-anadromous Arctic charr (Salvelinus alpinus) in Norway (Olstad *et al*., 2007a; Robertsen *et al*., 2007) and rainbow trout (*Oncorhynchus mykiss*) in Denmark (Jørgensen *et al*., 2007; Lindenstrøm *et al*., 2003).

There has been a long taxonomic/scientific debate on whether *Gyrodacytlus thymalli*, a species described from grayling (*Thymallus thymallus*), is a junior synonym of *G. salaris* (see e.g. Hansen *et al*., 2003; 2007a, 2007b; Meinilä *et al*, 2004, Fromm *et al*, 2014), and most evidence favours such a synonymisation. The National Center for Biotechnology Information (NCBI) has accepted a synonymisation of *G. salaris* and *G. thymalli* with the result that all accessions of DNA sequences previously assigned to *G. thymalli* are now assigned to *G. salaris*. Irrespective of this debate, strains isolated from grayling have never been found to be pathogenic to Atlantic salmon in experimental trials (see e.g. Sterud *et al*., 2002), and have not been observed on Atlantic salmon when in sympatry with grayling (Anttila *et al*., 2008). For the purpose of this chapter, *G. salaris* and *G. thymalli* are treated as two separate species.

2.1.2. Survival and stability off the host or in processed or stored samples

Survival of detached *G. salaris* is temperature dependent: approximately 24 hours at 19°C, 54 hours at 13°C, 96 hours at 7°C and 132 hours at 3°C (Olstad *et al*., 2006). *Gyrodactylus salaris* is known to survive between temperatures of 0°C to 25°C. Tolerance to temperatures above 25°C is unknown. *Gyrodactylus salaris* is sensitive to freezing and desiccation. It dies after a few days at pH≤5. It is more sensitive to low pH (5.1<pH<6.4) in association with aluminium and zinc than the host Atlantic salmon (Poleo *et al*., 2004; Soleng *et al*., 1999). For inactivation methods, see Section 2.4.5.

2.1.3. Survival and stability on host tissues

Survival of *G. salaris* attached to a dead host is temperature dependent: maximum survival times for *G. salaris* on dead Atlantic salmon are 72, 142 and 365 hours at 18°C, 12°C and 3°C, respectively (Olstad *et al*., 2006).

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with *G. salaris* according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) are: Arctic char (*Salvelinus alpinus*), Atlantic salmon (*Salmo salar*), brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*), grayling (*Thymallus thymallus*) and rainbow trout (*Oncorhynchus mykiss*).

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to infection with *G. salaris* according to Chapter 1.5. of the *Aquatic Code* are: none known.

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have identified *G. salaris* on the following organisms, but a long-term active infection has not been demonstrated: [Under study].

2.2.3. Likelihood of infection by species, host life stage, population or sub-populations

The prevalence and abundance of *G. salaris* on Atlantic strains of *S. salar* are higher than on other susceptible species and Baltic strains of *S. salar*. All life stages are susceptible, but prevalence and abundance on Atlantic salmon are highest in fry and parr stages, where mortality is also most likely to be observed.

For the purposes of Table 4.1 Atlantic salmon alevins and fry (e.g. up to approximately 1 g in weight) may be considered early life stages, parr and smolts can be considered as juveniles and all fish post smoltification as adults.

2.2.4. Distribution of the pathogen on the host

Gyrodactylus salaris usually occurs on the fins of infected Atlantic salmon, but the parasite distribution on the host may vary depending on intensity of infection (Jensen & Johnsen, 1992; Mo, 1992; Paladini *et al*., 2014). Parasites are also commonly found on the body but less commonly on the gills. On other hosts, the distribution may be different, but in general the parasite is relatively less abundant on the fins and relatively more common on the body compared with Atlantic salmon.

2.2.5. Aquatic animal reservoirs of infection

There are a number of combinations of host species and *G. salaris* strains which do not result in clinical signs of disease and may, therefore, act as reservoirs of infection. Several stocks of Atlantic salmon in the Baltic region are infected with *G. salaris* but do not generally show clinical signs or suffer mortality (Anttila *et al.,* 2008). *Gyrodactylus salaris* has been found in wild Arctic char without any observable signs or mortality (Robertsen *et al.,* 2007). Rainbow trout can be infected with some strains of *G. salaris* at a very low prevalence and abundance without observable signs (Paladini *et al.,* 2014).

2.2.6. Vectors

Gyrodactylus salaris parasites may attach themselves to any fish species not considered a susceptible species, for short periods of time. On some species limited reproduction takes place, but insufficient for the parasite to maintain a persistent infection (Paladini *et al.,* 2014). Thus, whilst any fish species could act as a vector, those on which reproduction occurs, are more likely to act as vectors. However, there is no evidence from the published literature that fish vectors have transmitted *G. salaris*.

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

Mortality in farmed Atlantic salmon fry and parr can be 100% if not treated. Mortality in wild Atlantic salmon fry and parr in Norwegian rivers can be as high as 98%, with an average of about 85% (Johnsen *et al*, 1999). Mortality in other susceptible species is usually low to negligible.

Prevalence in susceptible strains of Atlantic salmon reaches close to 100% in wild parr in rivers (Appleby & Mo, 1997); similarly, prevalence in farmed Atlantic salmon (in freshwater) rises to close to 100% within a short time after introduction of the parasite. Prevalence in resistant strains of Atlantic salmon in rivers and farms is likely to be low (Bakke *et al.,* 2007) and highly variable depending on season, location and age of the fish (Anttila *et al.,* 2008). Prevalence in other susceptible species is usually much lower than in Atlantic salmon and can be below 10% (e.g. in farmed rainbow trout; Buchmann & Bresciani, 1997).

2.3.2. Clinical signs, including behavioural changes

Wild Atlantic salmon with low infection intensities (one or up to a few tens) of *G. salaris* parasites usually do not exhibit any clinical signs. Increased parasite mean intensity over time often leads to increased flashing (fish scratch their skin on the substrate), increased mucous production (giving the fish a greyish appearance) and erosion of the fins.

Susceptible species other than Atlantic salmon usually only carry low numbers of *G. salaris* parasites and do not show clinical signs.

2.3.3. Gross pathology

Heavily infected Atlantic salmon may become greyish as a result of increased mucification, and at a later stage the dorsal and pectoral fins may become whitish as a result of increased thickness (mainly hyperplasia) of the epidermis. As the infestation continues, fish may have eroded fins, especially dorsal, tail and pectoral fins, because of parasite feeding. Secondary fungal infections (*Saprolegnia* spp.) are commonly observed in fish with infection with *G. salaris*.

2.3.4. Modes of transmission and life cycle

Gyrodactylus salaris is an obligate parasite with a direct life cycle. Parasites give birth to live offspring, and there are no other life stages. *Gyrodactylus salaris* can transfer to a new host via contact with live hosts, dead hosts, detached parasites in the water column, or parasites attached to the substrate.

Gyrodactylus salaris has spread between rivers and farms mainly by the translocation of live fish. Fish migrating through brackish water can also spread the parasite between neighbouring rivers (see also Section 2.3.5). The risk of transmission is greater between rivers located within the same brackish water system.

2.3.5. Environmental factors

Gyrodactylus salaris is a cold-water-adapted parasite and mainly lives in freshwater, reproducing normally at salinities up to 5–6 ppt (Malmberg, 1973; 1988). The average number of offspring per parasite peaks between 6.5°C and 13.0°C (Jansen & Bakke, 1991). At lower temperatures, *Gyrodactylus salaris* can survive longer in higher salinities (Soleng & Bakke, 1997). For example at 1.4°C, *G. salaris* may survive for 240 hours, 78 hours and 42 hours at 10 ppt, 15 ppt and 20 ppt salinity, respectively, while at 12°C it may survive for 72 hours, 24 hours and 12 hours at the same three salinities, respectively (Soleng & Bakke, 1997).

2.3.6. Geographical distribution

The original distribution of *Gyrodactylus salaris* is considered to be within the eastern parts of the Baltic area including the drainages of the Russian lakes Onega and Ladoga (Ergens, 1983; Malmberg & Malmberg, 1993). From these areas, the parasite has spread and it has been reported from several countries in Europe (Paladini *et al.,* 2021) in both wild and farmed populations. The parasite has been found on wild salmonids, mainly Atlantic salmon parr, in rivers in Finland, Norway, Russia and Sweden.

For recent information on distribution at the country level consult the WAHIS interface (https://wahis.oie.int/#/home).

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

Vaccines are not available.

2.4.2. Chemotherapy including blocking agents

Not applicable.

2.4.3. Immunostimulation

Immunostimulation is not available.

2.4.4. Breeding resistant strains

In laboratory experiments, selected breeding of Atlantic salmon has resulted in increased survival among the offspring (Salte *et al*., 2010). However, stocking rivers with resistant strains has not been attempted because the stock will remain infected and thus the parasite may spread to other rivers with susceptible hosts. In addition, stocking with resistant strains of Atlantic salmon (e.g. Baltic Neva strain) in affected rivers is not considered compatible with existing strain management of Atlantic salmon (i.e. preservation of the genetic integrity of wild stocks) (Karlsson *et al.*, 2019).

2.4.5. Inactivation methods

Gyrodactylus salaris is killed by exposure to water at 40°C for 5 minutes (Koski *et al.,* 2016) and to disinfectant (e.g. 1% Virkon S for 15 minutes) (Koski *et al.,* 2016), which may be used to eliminate transfer of the parasite with equipment.

2.4.6. Disinfection of eggs and larvae

Eggs that are transferred from infected farms should be disinfected (iodine-containing compounds have been used).

2.4.7. General husbandry

Gyrodactylus salaris is sensitive to changes in the chemical composition of the water. It is killed by the most commonly used chemicals for bath treatment of farmed salmon parr and eggs (e.g. high salinity salt water, formaldehyde and compounds containing chlorine or iodine) (Thrush *et al.,* 2019). Treatment of farmed salmonid populations with formaldehyde or other bath treatments will reduce the prevalence and abundance of *G. salaris* and may therefore render detection more difficult.

Gyrodactylus salaris is sensitive to acidic solutions (pH 5.0–6.0) of aluminium sulphate ($[Al_2(SO_4)_3]$) and zinc (Zn) (Poleo *et al*., 2004; Soleng *et al*., 1999). Aluminium sulphate is less toxic to fish than to *G. salaris* in moderately acidified waters, and has been used to eradicate the parasite from one river system in Norway (Pettersen *et al*., 2007). Recently, it was also found that *G. salaris* is sensitive to low doses of chlorine (Hagen *et al*. 2014).

3. Specimen selection, sample collection, transportation and handling

This section draws on information in Sections 2.2, 2.3 and 2.4 to identify populations, individuals and samples that are most likely to be infected.

3.1. Selection of populations and individual specimens

Sampling wild healthy populations should take place during the late summer or autumn or when the prevalence is known to be at its highest. Atlantic salmon should be targeted. In farms, fish showing clinical signs of infection (as described in Section 2.3.1) should be selected. Sampling should be avoided for a period after treatment for ectoparasites. In the absence of clinical signs, sampling of wild Atlantic salmon populations should target year class 1+ and 2+ as these are more likely of being infected than 0+ parr.

3.2. Selection of organs or tissues

Detection of *Gyrodactylus* and identification of *G. salaris* is a two-step process. Firstly, gyrodactylid parasite specimens are detected (e.g. on fish or fins) using optical equipment and picked off. Individual parasites are identified to species level using other equipment and methods.

Fish should be examined as whole specimens either live under anaesthesia (for example, with MS222), freshly killed, or preserved. In addition, fresh or preserved fins can be examined. Examination of live, anaesthetised fish is very time-consuming and not recommended. When Atlantic strains of Atlantic salmon parr are infected, almost all fish have at least one *G. salaris* on one of the fins. On some fish, *G. salaris* specimens may occur on the body or head, including the nostrils, the gills and the mouth cavity. The distribution of *G. salaris* on fins and other parts of the fish varies among fish species and strains of Atlantic salmon. For all hosts the examination of whole fish is recommended as it will increase the likelihood of detecting low intensity infections.

3.3. Samples or tissues not suitable for pathogen detection

Dead fish, stored on ice, are not acceptable for *Gyrodactylus* examination, even if the fish are kept separately in plastic bags, etc. The parasites die quickly if not covered in water and rapidly disintegrate.

3.4. Non-lethal sampling

Fish can be examined as live specimens under anaesthesia (for example, with MS222). Recently, a non-lethal method for isolating specimens of gyrodactylid parasites from fish was developed and tested on brown trout (Thrush *et al*., 2019). The method was shown to have a higher parasite recovery rate compared to whole body examination of killed fish (84.6% and 51.9%, respectively). The method has not yet been used on fish infected with *G. salaris,* but it is likely to be effective.

In addition, environmental DNA (eDNA) methods have been developed for detection of *G. salaris,* and its two main hosts, Atlantic salmon and rainbow trout, in water samples (Rusch *et al*., 2018). However, detection limits have not been established for these analyses.

3.5. Preservation of samples for submission

Fish should be killed immediately and should not be allowed to dry out before preservation. Whole fish should be preserved in 80–100% EtOH in bottles large enough to provide excess space and preservative. The concentration of EtOH after preservation should not be below 70%. As a rule of thumb this concentration is obtained if the proportion of fish tissue to EtOH does not exceed 1:9. If the concentration is lower, the mucous and epidermis may disintegrate and *Gyrodactylus* specimens, even if they are preserved, may drop off. Bottles should have an opening wide enough to avoid the possibility of scraping off *Gyrodactylus* specimens when fish are put into the bottle or when taken out for examination. Bottles should be stored in a horizontal position until the tissue is fixed/preserved to prevent the fish curling. When preservation of the fish is complete, the bottles can be stored in a vertical position.

As *G. salaris* is common on fins of Atlantic salmon, fins cut off from the body and stored in EtOH as described above can also be submitted. This is especially suitable for larger fish and under field conditions where, for example, transport is limited.

Formaldehyde-fixed *Gyrodactylus* specimens are difficult to identify morphologically and are also often unsuitable for DNA analysis.

3.5.1. Samples for pathogen isolation

Not applicable.

3.5.2. Preservation of samples for molecular detection

Tissue samples, i.e. isolated parasites, whole fish or fins, for PCR testing should be preserved in 70–90% (v/v) analytical/reagent-grade (absolute) ethanol. The recommended ratio of ethanol to tissue is 9:1 based on studies in terrestrial animal and human health. The use of lower grade (laboratory or industrial grade) ethanol is not recommended.

Template DNA should be prepared from live/fresh or EtOH-preserved specimens using a suitable DNA preparation protocol. DNA extraction kits may be used according to the manufacturers' instructions.

3.5.3. Samples for histopathology, immunohistochemistry or *in-situ* hybridisation

Not applicable.

3.5.4. Samples for other tests

Preservation of samples for environmental DNA (eDNA) analyses

Several methods for filtering water for eDNA analyses exist and the method has also been developed for the detection of G. salaris and its hosts, Atlantic salmon and rainbow trout (Rusch *et al*., 2018). In this method, duplicate water samples of 5 litres (2 × 5 litres) are collected and filtered on site on to glass fibre filters (47 mm AP25 Millipore, 2 µm pore size, Millipore, Billerica, USA) using a suitable pump, tubing and filter holder. Filters should be placed in separate zip-lock plastic bags containing silica gel and stored dry and dark until further analysis in the laboratory.

3.6. Pooling of samples

Sampled fish can be pooled, although each fish should subsequently be examined and analysed separately. Fins of fish from a farm or a river can be pooled and should also be examined and analysed separately, but in this instance each fin cannot be related to individual fish. Similarly, if fish are pooled for parasite removal using non-lethal bath methods (e.g. Thrush *et al.,* 2019), the parasites recovered cannot be related to individual fish.

Material from parasites should not be pooled for molecular diagnostic methods as data on the impact on diagnostic sensitivity and diagnostic specificity are not currently available.

4. Diagnostic methods

The methods currently available for identifying infection for surveillance (in healthy populations), presumptive and confirmatory diagnostic purposes are listed in Table 4.1. by life stage. The designations used in the Table indicate:

Key:

+++ =	Recommended method(s) validated for the purpose shown and usually to stage 3 of the OIE Validation Pathway; OIE recommended method(s) will be mentioned in the text;
++ =	Suitable method(s) but may need further validation;
+ =	May be used in some situations, but cost, reliability, lack of validation or other factors severely limits its application;
Shaded boxes =	Not appropriate for this purpose.

The selection of a test for a given purpose depends on sensitivity, specificity, repeatability and reproducibility. OIE Reference Laboratories welcome feedback on diagnostic performance for assays, in particular PCR methods, for factors affecting assay sensitivity or specificity, such as tissue components inhibiting amplification, nonspecific or uncertain bands, etc., and any assays that are in the +++ category.

Table 4.1. *OIE recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals*

Method	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis			
	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV
Morphological examination		+	+	1		+	+	1				
Histopathology[3]												
Cytopathology[3]												
Cell culture												
Real-time PCR (using parasite sample)		+	+	1		+	+	1				
ddPCR/Real-time PCR (using environmental sample)	+			1								
Conventional PCR		+	+	1		+	+	1		++	++	2
Amplicon sequencing[4]										++	++	2
***In-situ* hybridisation**												
Bioassay												
LAMP												
Ab-ELISA												
Ag-ELISA												

LV = level of validation, refers to the stage of validation in the OIE Pathway (chapter 1.1.2); PCR = polymerase chain reaction; ddPCR = droplet digital PCR;
LAMP = loop-mediated isothermal amplification; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively;
[1]For confirmatory diagnoses, methods need to be carried out in combination (see Section 6).
[2]Early and juvenile life stages have been defined in Section 2.2.3.
[3]Histopathology and cytopathology can be validated if the results from different operators has been statistically compared.
[4]Sequencing of the PCR product.
Shading indicates the test is inappropriate or should not be used for this purpose

4.1. Parasite detection

Live anaesthetised fish, freshly cut fins or EtOH-preserved fish or fins should be examined under a binocular dissecting microscope with good illumination. The fish should be placed in a box and completely covered in freshwater. Preserved fish can also be examined in EtOH. Living parasites are more easily detected by their movements, thus disturbing light refraction on the skin of the fish should be avoided. Live *Gyrodactylus* are colourless while EtOH-preserved *Gyrodactylus* specimens are usually slightly opaque. Dark field illumination microscopy will increase the contrast and the parasites will be detected more easily. The whole surface of the fish, including gills and mouth cavity, must be examined. It is best to use two forceps for this process. The fins of relatively small fish, usually less than 10 cm, can also be studied using illumination through the bottom of the microscope stage, which makes *Gyrodactylus* specimens easy to observe.

A non-lethal method (Thrush *et al.*, 2019) results in the collection of ectoparasites from the treated fish on filter paper. The filter can then be screened for the presence of parasites using a stereomicroscope.

Once individual gyrodactylid parasites have been visualised, they can be removed from the fish, fins or filter paper using a pipette. The species of gyrodactylid can be determined using one of the tests described in this section.

4.2. Morphological examination

Morphological identification of *Gyrodactylus* species is based on the morphology and morphometry of marginal hooks anchors (hamuli) and bars in the haptor (the attachment organ). Good preparation of specimens is a prerequisite for species identification. Morphological identification is only recommended for preliminary diagnosis of *G. salaris* and should not be used for confirmation, for which molecular methods are recommended.

Digestion of the soft tissue, leaving the hard parts only, is recommended when high-resolution morphometrics is required for reliable morphometric diagnosis. The soft tissue can be digested in a solution (approx. 1 µl) of 75 mM Tris, 10 mM EDTA (ethylene diamine tetra-acetic acid), 5% SDS (sodium dodecyl sulphate) and 100 mg ml^{-1} proteinase K, pH 8.0. After adding the digestion solution, the reaction should be monitored microscopically until completion and then ended by adding a stop solution (1:1 glycerol and 10% neutral buffered formalin). The procedure for digestion is described in detail in Harris *et al*., 1999. Identification of *G. salaris* should be in accordance with references: Cunningham *et al*., 2001; Malmberg, 1957; 1970; McHugh *et al*., 2000; Olstad *et al*., 2007b; Shinn *et al*., 2004.

The size of the haptoral hard parts in *Gyrodactylus* varies extensively with, for example, temperature, whereas shape is more stable (see e.g. Mo, 1991). The capability of linear measurements to capture morphology might therefore not always be sufficient for reliable diagnosis (Olstad *et al*., 2007b).

Gyrodactylus salaris can be differentiated from other *Gyrodactylus* species by trained morphologists on the basis of morphology but not from *G. thymalli* (Olstad *et al*., 2007b; and see Section 2.1.1). In addition, *G. salaris* is morphologically similar to *Gyrodactylus teuchis* from brown trout, Atlantic salmon, and rainbow trout, but can be differentiated by trained morphologists on the basis of the shape of the marginal hook sickle. *Gyrodactylus teuchis* has a longer and more constantly curved sickle blade (see Cunningham *et al*., 2001).

4.3. Histopathology and cytopathology

Not applicable.

4.4. Cell culture for isolation

Not applicable.

4.5. Nucleic acid amplification

For all molecular tests below DNA can be extracted using standard DNA extraction kits.

4.5.1. Real-time PCR

Both real-time PCR (Collins *et al*., 2010) and digital droplet (dd) PCR (Rusch *et al*., 2018) have been developed for *G. salaris*. Real-time PCR has not been widely applied for diagnostics of *G. salaris,* and ddPCR is developed for use in connection with eDNA-methods. Both these methods target the ribosomal internal transcribed spacers region (ITS) and have the same diagnostic limitations (see below and Section 4.5.2). However, real-time PCR is faster than conventional PCR and DNA sequencing (Section 4.4.2) and can be applied as a fast means to exclude species other than *G. salaris/G. thymalli*, and the method is therefore mentioned briefly here. Conventional PCR and sequencing of the mitochondrial cytochrome oxidase 1 (CO1) gene (Sections 4.4.2 and 4.5.2), can then be performed on those species

with a positive result from real-time PCR for haplotype identification, which will allow *G. salaris* to be differentiated from *G. thymalli* (4.6.2).

The real-time PCR assay of Collins *et al.* (2010) is a TaqMan minor groove binder (MGB) real-time PCR assay that targets a 60 bp unique sequence motif in the ITS1 region of *G. salaris*/*G. thymalli*. It applies the forward primer F (5'-CGA-TCG-TCA-CTC-GGA-ATC-G-3'), reverse primer R (5'-GGT-GGC-GCA-CCT-ATT-CTA-CA-3') and TaqMan MGB probe Gsal2 (5'-FAM-TCT-TAT-TAA-CCA-GTT-CTG-C-3') labelled with the fluorescent reporter dye FAM at the 5'-end and a non-fluorescent quencher MGBNFQ at the 3'-end. Amplifications were performed in a total volume of 20 µl containing TaqMan Universal PCR Master mix (with UNG; Applied Biosystems), 0.9 µM of each forward and reverse primer and 0.25 µM of each probe and dH2O (Sigma) to a final volume of 20 µl. One µl of lysate from a parasite specimen was added to the each test tube. The cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes followed by 35 cycles of 95°C for 15 seconds and 60°C for 1 minute and run in an ABI 7000 Sequence Detection System (Applied Biosystems). The efficiency of the singleplex assay was reported as ranging from 93.1% to 101.1% and the limit of detection (dilution of the crude *Gyrodactylus spp.* lysate) was 10^{-4}. Further details can be found in Collins *et al.* (2010).

4.5.2. Conventional PCR

Analysis of the ribosomal RNA gene internal transcribed spacer region (*ITS*)

For amplification of a 1300 base pair product of the ITS-region, covering ITS1, 5.8S, and ITS2, primers, such as 5'-TTT-CCG-TAG-GTG-AAC-CT-3' and 5'-TCC-TCC-GCT-TAG-TGA-TA-3', may be used. The cycling conditions for PCR are as follows, initial denaturation at 95°C for 5 minutes; 30 cycles of 94°C for 1 minute, 50°C for 1 minute, 72°C for 2 minutes; final extension at 72°C for 7 minutes (Cunningham, 1997). If partially degraded material is analysed or if the PCR above does not give a positive result, the ITS1 and ITS2 spacers can be amplified in two separate reactions using primer sets and PCR conditions described in Matejusová *et al*., 2001. The amplification of ITS2 alone, using the primers 5'-CAT-CGG-TCT-CTC-GAA-CG-3' and 5'-TCC-TCC-GCT-TAG-TGA-TA-3' and using the same protocol as above is sufficient.

The primers for amplification of ITS are not specific to G. salaris and will amplify all or most species of Gyrodactylus. Positive PCR products should thus be sequenced to identify the haplotype, which can be used for species confirmation (see Section 4.5).

Analysis of the mitochondrial cytochrome oxidase I (*CO1*) *gene*

For amplification of the CO1-gene, the primers 5'-ATA-TAG-ACG-ATT-TGT-TTT-CA-3' and 5'-ACA-GAT-TAC-TTG-GTA-TTA-CA-3' (Kuusela *et al*., 2009) may be used to amplify the full-length gene (1600 base pairs) which is recommended. The primers 5'-TAA-TCG-GCG-GGT-TCG-GTA-A-3' and 5'-GAA-CCA-TGT-ATC-GTG-TAG-CA-3') (Meinilä *et al*., 2002) may be used to amplify a 800 base pairs fragment if the first PCR is unsuccessful. The cycling conditions for both PCRs are as follows, initial denaturation at 95°C for 5 minutes; 35 cycles of 95°C for 1 minute, 50°C for 1 minute, 72°C for 2 minutes; final extension at 72°C for 7 minutes. Additional primer sets for amplification of CO1 can be found in references: Hansen *et al*., 2003; Kuusela *et al*., 2009; Meinilä *et al*., 2002; 2004.

Primers recommended for amplification of CO1 may not be specific for G. salaris and may not amplify all isolates. Positive PCR products should be sequenced to identify the haplotype (Section 4.6).

The following controls should be run with each assay: negative extraction control; positive control; no template control.

4.5.3. Other nucleic acid amplification methods

Not applicable.

4.6. Amplicon sequencing

4.6.1. ITS sequencing and sequence analysis

Amplified ITS fragments prepared as in Section 4.4.2 should be sequenced using the PCR primers and, in addition, internal sequencing primers (Cunningham, 1997; Matejusová *et al.*, 2001) should be used to obtain overlapping reads of each nucleotide. The resulting ITS sequences should be subjected to a BLAST search in GenBank/EMBL to establish identity with known sequences. Several sequences of other species infecting salmonids, e.g. *G. derjavini*, *G. derjavinoides*, *G. truttae*, and *G. teuchis* are available in GenBank/EMBL. *G. thymalli* cannot be distinguished from *G. salaris* by this method, but sequences of ITS distinguishes *G. salaris* from all other known species. Host identity of sequences in GenBank/EMBL should thus always be checked, however, GenBank has synonymised *G. salaris* and

G. thymalli. Therefore, if the BLAST search of the ITS sequences identifies the parasite as *G. salaris*, CO1 sequencing and sequence analysis are recommended to identify the haplotype in question (Section 4.6.2).

4.6.2. CO1 sequencing and sequence analysis

Amplified CO1 fragments prepared as described in Section 4.5.2 should be sequenced using the PCR primers and, in addition, internal sequencing primers (Kuusela *et al*., 2009; Meinilä *et al*., 2002) should be used to obtain overlapping reads of each nucleotide. The resulting CO1 sequences should be subjected to a BLAST search in GenBank/EMBL to identify the haplotype.

If the obtained sequence does not have a 100% match in GenBank/EMBL, a phylogenetic analysis can be performed to establish the relationship to other available sequences. Different haplotypes and clades of *G. salaris* and *G. thymalli* can be distinguished with this method. CO1 sequences can be used to assign specimens to a haplotype or clade and thus infer the identity as *G. salaris* or *G. thymalli*. Clades (i.e. groups of haplotypes with a common ancestor) of *G. salaris* generally correspond well to host preferences or the geographical distribution of the parasites, with a few exceptions, and some strains, as defined by CO1-sequences (haplotypes), are known to be pathogenic to Atlantic salmon.

GenBank has synonymised *G. salaris* and *G. thymalli*, with the result that all accessions previously listed as *G. thymalli* are now *G. salaris;* the haplotypes in Table 4.6.2 can be retrieved from GenBank. Table 4.6.2 assigns the haplotypes to either *G. salaris* or *G. thymalli,* to support identification of *G. salaris* based on CO1 sequencing (new haplotypes should be compared to the nearest known relative). In rivers where both grayling and Atlantic salmon are found, establishing the *G. thymalli* haplotypes present on grayling will support any subsequent monitoring for *G. salaris* on Atlantic salmon.

Table 4.6.2 Gyrodactylus salaris *and* G. thymalli *GenBank accession numbers for CO1 nucleotide sequences*

G. salaris*							***G. thymalli****		
AF479750	AY146602	AY258354	AY486492	AY486517	AY486542	EU186166	AF540893	AY486545	DQ159928
AF540891	AY146603	AY258355	AY486493	AY486518	AY486543	EU186167	AF540894	AY486546	DQ180333
AF540892	AY146604	AY258356	AY486494	AY486519	AY840222	EU186168	AF540895	AY486547	DQ993195
AF540904	AY146605	AY258357	AY486495	AY486520	AY840223	EU186169	AF540896	AY486548	EF495063
AF540905	AY146606	AY258358	AY486496	AY486521	DQ468128	EU186170	AF540897	AY486549	EF527269
AF540906	AY146607	AY258359	AY486497	AY486522	DQ517533	EU186171	AF540898	AY486550	EF612464
AF542161	AY146614	AY258360	AY486498	AY486523	DQ778628	EU186172	AF540899	AY486551	MG273445
AF542162	AY258336	AY258361	AY486499	AY486524	DQ923578	EU186173	AF540900	AY486552	MG273446
AF542163	AY258337	AY258362	AY486500	AY486525	DQ988931	EU186174	AF540901	AY486553	MG273447
AF542164	AY258338	AY258363	AY486501	AY486526	DQ993189	EU186175	AF540902	AY840224	MG273448
AF542165	AY258339	AY258364	AY486502	AY486527	DQ993190	EU186176	AF540903	DQ159913	
AF542166	AY258340	AY258365	AY486503	AY486528	DQ993191	EU186177	AF542167	DQ159914	
AY146589	AY258341	AY258366	AY486504	AY486529	DQ993192	EU223246	AF542168	DQ159915	
AY146590	AY258342	AY258367	AY486505	AY486530	DQ993193	EU304825	AF542169	DQ159916	
AY146591	AY258343	AY258368	AY486506	AY486531	DQ993194	GQ129460	AF542170	DQ159917	
AY146592	AY258344	AY258369	AY486507	AY486532	EF117889	GQ129461	AF542171	DQ159918	
AY146593	AY258345	AY258370	AY486508	AY486533	EF524576	GQ129462	AY146608	DQ159919	
AY146594	AY258346	AY258371	AY486509	AY486534	EF524577	GQ129463	AY146609	DQ159920	
AY146595	AY258347	AY258372	AY486510	AY486535	EF524578	GQ370816	AY146610	DQ159921	
AY146596	AY258348	AY258373	AY486511	AY486536	EF570120	GU187354	AY146611	DQ159922	
AY146597	AY258349	AY258374	AY486512	AY486537	EU186161	KJ941020	AY146612	DQ159923	
AY146598	AY258350	AY486488	AY486513	AY486538	EU186162	KT344124	AY146613	DQ159924	
AY146599	AY258351	AY486489	AY486514	AY486539	EU186163	KT344125	AY472084	DQ159925	
AY146600	AY258352	AY486490	AY486515	AY486540	EU186164	KT344126	AY472085	DQ159926	
AY146601	AY258353	AY486491	AY486516	AY486541	EU186165	KT344127	AY486544	DQ159927	
						KT344128			

*Note: *G. salaris* and *G. thymalli* have been synonymised by NCBI GenBank, i.e. all accessions previously listed as *G. thymalli* are now *G. salaris*.

Where the sequence is not assigned to one of the recognised haplotypes (CO1 sequences) of *G. salaris* or *G. thymalli* advice should be sought from the OIE Reference Laboratory. The OIE Reference Laboratory will keep an updated database of CO1-sequences and will assist in the diagnosis. It is recommended that the OIE Reference Laboratory is informed of any significant detections of *G. salaris* and *G. thymalli* in order to confirm the cases.

4.7. *In-situ* hybridisation

Not applicable.

4.8. Immunohistochemistry

Not applicable.

4.9. Bioassay

Not applicable.

4.10. Antibody- or antigen-based detection methods

Not applicable.

4.11. Other methods

Not applicable.

5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations

Real-time PCR is the recommended test for surveillance to demonstrate freedom of disease in apparently healthy populations. Sequencing of the amplified CO1 amplicon is required for confirmation of infection in any parasite that identified as positive by PCR.

6. Corroborative diagnostic criteria

All suspect positive samples of *G. salaris* from country or zone or compartment considered free from infection with *G. salaris* should be referred immediately to the OIE Reference Laboratory to definitively identify the parasite based on the most up-to-date information (see Section 4.6). Submissions should be made whether or not clinical signs have been observed.

This section only addresses the diagnostic test results for detection of infection in the absence (Section 6.1) or presence of clinical signs (Section 6.2) but does not evaluate whether the infectious agent is the cause of the clinical event.

The case definitions for a suspect and confirmed case have been developed to support decision making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent.

6.1. Detection in apparently healthy animals or animals of unknown health status[1]

Healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link(s) to an infected population. Geographic proximity to, or movement of animals or animal products or equipment, etc., from a known infected population equate to an epidemiological link. Alternatively, healthy populations will be sampled in surveys to demonstrate disease freedom.

1 For example, transboundary commodities.

6.1.1. Definition of suspect case in apparently healthy animals

The presence of infection with *G. salaris* shall be suspected if at least one of the following criteria is met:

i) Identification of *G. salaris* by morphological examination;

ii) A positive result by real-time PCR;

iii) A positive result by ddPCR or real-time-PCR using an environmental sample.

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with *G. salaris* is considered to be confirmed if the following criterion is met:

i) A positive result by conventional PCR testing of parasite samples and sequencing of one or both of the ITS fragments and the CO1 fragment. The ITS sequences obtained are then analysed according to Section 4.6.1 and the CO1sequences according to Table 4.6.2 (see Section 4.6.2).

6.2. Clinically affected animals

Clinical signs are not pathognomonic for a single disease; however, they may narrow the range of possible diagnoses.

6.2.1. Definition of suspect case in clinically affected animals

The presence of infection with *G. salaris* shall be suspected if at least one of the following criteria is met:

i) Gross pathology or clinical signs associated with the disease as described in this chapter, with or without elevated mortality;

ii) Identification of *G. salaris* by morphological examination;

iii) A positive result by conventional PCR;

iv) A positive result by real-time PCR.

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with *G. salaris* is considered to be confirmed if the following criterion is met:

i) A positive result by conventional PCR testing of parasite samples and sequencing of one or both of the amplified ITS fragments and the CO1 fragment. The ITS sequences obtained are then analysed according to Section 4.6.1 and the CO1 sequences according to Table 4.6.2 (see Section 4.6.2).

6.3. Diagnostic sensitivity and specificity for diagnostic tests: under study

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with *G. salaris* is provided in Table 6.3.1. (note: no data are currently available). This information can be used for the design of surveys for infection with *G. salaris*, however, it should be noted that diagnostic performance is specific to the circumstances of each diagnostic accuracy study (including the test purpose, source population, tissue sample types and host species) and diagnostic performance may vary under different conditions. Data are only presented where tests are validated to at least level two of the validation pathway described in Chapter 1.1.2 and the information is available within published diagnostic accuracy studies.

Table 6.3.1. *Diagnostic performance of tests recommended for surveillance or diagnosis*

Test type	Test purpose	Source population	Tissue/ sample type	Species	DSe (*n*)	DSp (*n*)	Reference test	Citation
Real-time PCR	Surveillance	–	Parasites	–	Not yet available	Not yet available	–	–
Amplicon sequencing	Diagnosis	–	Parasites	–	Not yet available	Not yet available	–	–
Morphological examination	Diagnosis	–	Parasites	–	Not yet available	Not yet available	–	–

DSe = diagnostic sensitivity; DSp = diagnostic specificity; *n* = number of samples used in the study.

7. References

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* *

NB: There is an OIE Reference Laboratory for infection with *Gryodactylus salaris*
(see Table at the end of this *Aquatic Manual* 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/).
Please contact the OIE Reference Laboratories for any further information on infection with *G. salaris*.

NB: FIRST ADOPTED IN 1997 AS GYRODACTYLOSIS OF ATLANTIC SALMON (*GYRODACTYLUS* F).
MOST RECENT UPDATES ADOPTED IN 2021.