6.1. Annex A

Guidelines to reviewers

Guidelines were issued in March 2009 and revised in July 2009. In the event, the method suggested below for the data transfer sheet was not followed, and a straight copy was made of the data entry sheet for corrections, codings and interpretations to be inserted.

Method of data collection - Issue 2

Guidelines for reviewers

The proforma is implemented as an Excel workbook, but the data and metadata can be collected by handsearchers in any tabular file format. The data fields have been circulated previously.

One aspect is to build up - or obtain - a glossary of controlled vocabulary. Please jot down any words, phrases or acronyms that you find that might need documenting for a non-specialist or future audience.

The method of handsearching involves looking systematically for any sources of information. Your own professional database is an excellent starting point, but needs to be cross-checked against other search engines to demonstrate lack of bias. To an external auditor, you need to demonstrate, for example, that poor quality reports have been seen and excluded for good reasons – just not having them in your database may indicate prejudice.

A) Metadata sheet

1) **Select database(s)** to search for records of outbreaks.

2) **Record** as metadata: the name of database, the search terms used on each occasion, and date(s) of search(es).
   Treat distinct searches of same database as separate. You will probably download the results of the search (ie list of references) and store them in your local, personal, **bibliographic** database (Endnote, RefMan, etc).

3) **Identify** from keywords, titles, or abstracts which reports are worth further investigation. Decide if others are irrelevant (spurious products of the keywords) and can be discarded completely, or if they are interesting but not appropriate for the current project so should be retained but flagged as not used. That would include poor-quality or discredited reports.

4) Desirable, but not essential, to **obtain** the selected items as PDF or other electronic format, so you have a definitive copy from which to work. If working from a paper copy to hand, try to arrange for it to be scanned and stored. (Cochrane organization does not specify if these are collected centrally, but it would add value to the database if copies were retained.)

5) For grey literature, create a **bibliographic entry** and take a copy of the electronic source, noting the day of copying.
6) Using the convention for your personal database, **allocate a citation reference** to each paper (source). This links one or more rows of extracted data to the source in your bibliography.

B) Data Entry sheet

7) From each source, **extract the factual information** about a disease occurrence into the data table as a row. Enter multiple values of the same type of information separated by semi-colons (;). If a ; appears in the text you need to copy (substitute : or /). Whenever possible use the actual words (and spelling) in the source. DO NOT supply your own "corrections" but do indicate these in the interpretation and comment fields.

8) **Each row** of the data table should record an **outbreak** or **occurrence**. An outbreak is a discrete event; an occurrence is a continuous presence One paper may list several outbreaks; each outbreak should therefore be extracted as a separate row with the same citation for each. For review papers, when possible, go back to the report that the review is citing, as more primary. Conversely, one outbreak may have been reported in several sources (first report, confirmation, discussion). In that situation list all the citations, separated by ;s. (As a pragmatic rule, an "outbreak" has a start and an end. Sources that describe several periods or waves of infection at the same location should be treated as more than one outbreak.)

9) The objective is to **extract structured information** from sources that may be less structured. If a report is lacking some required fields but otherwise appears genuine, record "missing" (or n/a) in the cell of the table. It may be possible to contact the author or NRL and obtain the extra information, which would then be cited as "pers comm" or other.

10) Advice for what to enter in each field is documented in the **codebook** sheet. Everyone should work from the same rules but errors or improvements to the codebook must be allowed for in the initial stages of the project.
If in doubt, use your own judgment and note this under "General comments".

11) Add your **interpretation and assessment** in the comment fields.

12) **Record** your **search** progress in the metadata sheet.

13) At appropriate intervals, or when the search is deemed complete, **send** (copies of) the datasheet, metadata sheet, bibliography, and source PDFs to the project coordinator.

C) Data transfer sheet

14) This sheet starts with two copies of each value from the Data Entry sheet. Values in the pink columns cannot be over-written. Values in the white columns can be overwritten with codes or interpretations of the original data.

15) The EFSA database can then contain fields for both the raw data values and the codes used for structuring and displaying the data.

6.2. Annex B

Disease Summary Reports

1) Template used by reviewers

2) Fish diseases

- EHN
- EUS
- IHNV
- ISA
- KHV
- VHS

3) Mollusc diseases

- Infection with *Bonamia exitiosa*
- Infection with *Bonamia ostrea*
- Infection with *Marteilia refringens*
- Infection with *Mikrocytos mackini*
- Infection with *Perkinsis marinus*

4) Crustacean diseases

- Taura syndrome
- White Spot syndrome
- Yellowhead disease

1) Review template

Critical review of aquatic disease [X]: disease detection, pathogen identification and typing

Review author(s) & Date:

[names & addresses]

Causative agent description:

Characteristics of causative agent
Evidence for strain differentiation

Summary of methods findings:

Very short summaries of best practice for diagnosis (in field and lab confirmation, name of method only+ref for details).

Gaps in knowledge

eg limitations of method, special cares needed, sensitivity/specificity, perceived problems and possible confusions)

Critical review of aquatic disease [X]: occurrence and distribution

Review author(s) & Date:

[names & addresses]

Selection of studies:

[Where searched; search terms and when; criteria for retaining/discarding found items. List of criteria used in quality ratings.]

Data and analyses:

[Number] source documents were located and are listed in [bibliography filename] with extracted data tabulated in [table filename].

Summary of disease findings:

What/where/when. Overview of host species (if >10 spp, perhaps abbreviate to "many species from families ..."), refer to table 1 for list. Geographical spread of outbreaks or general occurrence, refer to table 2 for list. Range over time, apparent patterns or trends. Gaps in knowledge (eg lack of information from some countries), hazard rating.

Mortalities and importance:

Chance to comment on the effects and importance to aquaculture/environment of the disease.

Possible sources of bias:

Any comments on availability of material, selectivity of reporting, reasons why the published material may not be complete or representative of the distribution of the disease.

Other comments [if any]:

eg, identified after outbreaks or by routine surveillance, control measures, economic impacts, etc.

[tables]

2) Fish diseases

Epizootic haematopoietic necrosis (EHN): disease detection, pathogen identification and typing

Review author(s) & Date:

Dr B Hill,

Centre for Environment, Fisheries and Aquaculture Science, Weymouth, United Kingdom

October 2009

Causative agent description:

Characteristics of causative agent

The causative agent of EHN is an iridovirus, epizootic haematopoietic necrosis virus (EHNV), a member of Genus *Ranavirus in* Family *Iridoviridae*. Viruses within the genus are referred to as ranaviruses, isolates of which have been obtained from a wide variety of fish, amphibians and reptiles and some are highly pathogenic. Ranavirus virions are large (150-180 nm diameter) with with a double-stranded DNA genome (150-170 kbp) within an icosahedral capsid and a lipid outer membrane. The virus replicates both in the nucleus and in the cytoplasm with virus assembly taking place in the cytoplasm. All currently known ranaviruses are closely related; they strongly cross react antigenically and can only be differentiated from each other by genome sequencing or endonuclease analysis (Ahne et al., 1998; Hyatt et al., 2000; Marsh et al., 2002). Two other fish ranaviruses, European catfish virus (ECV) and European sheatfish virus (ESV), which cause a systemic necrotising disease in wild and farmed fish in Europe, were for some years thought to be strains of EHNV but the genome sequencing work of Hyatt et al. (2000) led the authors to conclude that ECV and ESV are distinct from EHNV and that ECV and ESV are probably variants of the same virus.

Evidence for strain differentiation

There is no published or other source of evidence known to the reviewer for the existence of different genotypes or serotypes of EHNV.

Summary of methods findings:

Although originally isolated in RTG-2 cells (Langdon et al. 1986; Langdon and Humphrey, 1987)), EHNV grows better in many other fish cell lines, including BF-2, FHM and CHSE-214, at temperatures between 15 and 22°C (Crane et al. 2005). According to the OIE Reference Laboratory for EHN, isolation of EHNV in tissue culture followed by virus identification cells remains the gold standard for diagnosis of the disease, with use of BF-2 cells at 22°C being preferred – see Chapter 2.3.1 in the OIE Aquatic Manual (OIE, 2009). Identification of EHNV isolated in tissue culture was initially achieved by electron microscopy (EM) alone (Langdon et al, 1986; Langdon and Humphrey, 1987) but this method only reveals ranavirus-like particles and so is not specific for EHNV. It can be made more specific by immuno-staining such as the immunogold method. An alternative approach for identification of EHNV in infected fish tissue sections or infected tissue cultures is use of virus antigen detection methods such as immunofluorescence and immunoperoxidase staining. The virus can also be detected in tissue culture or directly in homogenates of infected fish tissues by an antigen capture ELISA method using rabbit anti-EHNV antibodies (Whittington and Steiner, 1993). However, in all these antigen detection methods using polyvalent antisera, positive reactions are not specific for EHNV as there is cross-reaction with all other ranaviruses, so should be regarded only as indicative and supporting evidence for EHN disease or EHNV is essential for a confirmatory diagnosis. Confirmation of the presence or isolation of EHNV is best achieved by polymerase chain reaction (PCR) using primers based on the MCP gene and restriction

enzyme analysis (REA) or sequencing of the PCR product, as described in Chapter 2.3.1. of the OIE Aquatic Manual (OIE, 2009). This differentiates EHNV from the closely related ECV or ESV viruses, and all other ranaviruses, and is the only method available for final confirmatory diagnosis of EHN.

Gaps in knowledge

The strong antigenic cross reactivity between all ranaviruses when using polyvalent rabbit or sheep antisera limits the use of simple and convenient antigen detection methods such as ELISA, IFAT and immunoperoxidase for confirmatory diagnosis of EHN. It is possible that this limitation could be overcome by development and use of monoclonal antibodies (Mabs) specific to EHNV. Such Mabs might also reveal whether there is more than one antigenic type of EHNV or whether all the isolates in Australia are identical. It would also be particularly useful if the monoclonal antibodies allowed clear differentiation of EHNV from ECV and ESV so that should the virus get introduced into European fish populations it could be identified quickly by simple rapid tests such as ELISA. At the genetic level, it is not clear whether all the isolates in Australia are identical and therefore likely to have a common origin, possibly a single introduction from another country or a single emergence of a virulent strain from a normally non-pathogenic ranavirus present in one or more aquatic animal species in south eastern Australia. Further sequencing of the genome of several isolates of geographically dispersed isolates of EHNV is needed to determine this. The real-time PCR assay developed by Pallister et al. (2007) is useful as an initial screening method but with further development it could become a sensitive and specific method for detecting EHNV and differentiating it from other ranaviruses, particularly ECV/ESV.

References

Ahne, W., Bearzotti, M., Bremont, M. & Essbauer, S. (1998). Comparison of European Systemic Piscine and Amphibian Iridoviruses with Epizootic Haematopoietic Necrosis Virus and Frog Virus 3. *Journal of Veterinary Medicine, Series B* **45**, 373-383.

Crane, M. S. J., Young, J. & Williams, L. M. (2005).Epizootic haematopoietic necrosis virus (EHNV): Growth in fish cell lines at different temperatures. Bulletin of the European Association of Fish Pathologists 25, 228-231.

Hyatt, A. D., Gould, A. R., Zupanovic, Z., Cunningham, A. A., Hengstberger, S., Whittington, R. J., Kattenbelt, J. & Coupar, B. E. H. (2000). Comparative studies of piscine and amphibian iridoviruses. Archives of Virology 145, 301-331.

Langdon J.S., Humphrey J.D., Williams L.M., Hyatt A.D. & Westbury H.A. (1986). First virus isolation from Australian fish: an iridovirus-like pathogen from redfin perch, Perca fluviatilis L. Journal of Fish Diseases, 9, 263–268.

Marsh, I. B., Whittington, R. J., ORourke, B., Hyatt, A. D. & Chisholm, O. (2002). Rapid differentiation of Australian, European and American ranaviruses based on variation in major capsid protein gene sequence. Molecular and Cellular Probes 16, 137-151.

OIE (2009). Manual of Diagnostic Tests for Aquatic Animals. Chapter 2.3.1. Epizootic haematopoietic necrosis. Accessed at: http://www.oie.int/eng/normes/fmanual/2.3.01_EHN.pdf

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Epizootic haematopoietic necrosis (EHN): occurrence and distribution

Review author(s) & Date

Dr B Hill,

Centre for Environment, Fisheries and Aquaculture Science, Weymouth, United Kingdom

October 2009

Selection of studies

Searches for published and unpublished studies and other sources of information on EHN were conducted on several occasions during 2009. ASFA, ProQuest Deep Indexing: Aquatic Sciences and Scopus: Natural Sciences were the main scientific publications databases searched. Search terms included: EHN, EHNV, redfin perch virus, and epizootic haematopoietic necrosis. Searches were also made of ProMED mail and Google scholar for other sources of information. On-line disease databases searched included OIE World Animal Health Information Database (WAHID) for 2005-present, OIE HandiSTATUS II for 1996-2004, OIE Regional Aquatic Animal Disease Reports (Asia-Pacific region) for 2002-2009, and the Defra/OIE International Database on Aquatic Animal Diseases (www.collabcen.net) for all first reports of occurrence by location or by host. All relevant data was compared with the occurrence and distribution information for EHN in the OIE Manual of Diagnostic Tests for Aquatic Animals (2009) and the EFSA report on aquatic species susceptible to diseases listed in Directive 2006/88/EC' (EFSA 2008).

Printed copies were obtained for all relevant publications. Only documents that contained original information on detection of the disease and/or the pathogenic agent were critically reviewed.

Data and analyses

A total of 44 references for publications were obtained by searches for epizootic haematopoietic necrosis, EHN and EHNV. Of these, 15 were disregarded after finding they did not contain any relevant information on EHN or EHNV, leaving 29 publications for review. Of the 29 publications, only 7 contained original data on occurrence of EHN by location or by host: these were selected for critical review and these are listed in the file 'Publications critically reviewed for EHN occurrence data' and the extracted data, together with the data quality assessments, are tabulated in the Excel file 'EHN-review-final'.

The quality ratings applied to the evidence presented for occurrence were:

- **Definite**: full details of identification methods were given or confirmation came in a subsequent study; validated methods used. Convincing evidence.
- **Probable**: Most details of identification were given; validated methods used but firm confirmation not achieved. Look very likely but some uncertainties
- **Possible**: Few details of identification given and/or non-validated or indirect methods used. Grounds for a suspect case only.
- **Doubtful**: Scant or no test details given and/or non-specific methods used. Major lack of supporting evidence.

Summary of disease findings

Initially detected in wild redfin perch populations subject to mass mortalities in Victoria in Australia in 1984, and referred to as 'an iridovirus-like pathogen' (Langdon et al. 1986), EHNV was the first virus of fish to be found in Australia. In a subsequent publication (Langdon et al. 1987), the disease was named EHN. It soon became clear that outbreaks were common in redfin perch during the summer months in Victoria. It was later detected for the first time in cultured rainbow trout (Langdon et al. 1988) in New South Wales in Australia (Whittington et al. 1994 and Whittington et al. 1999). A few years later, there was a 'grey literature' first report of mortalities caused by EHN in wild populations of redfin perch in South Australia (Pierce et al. 1991) and this was subsequently confirmed by Whittington et al. (1996). Further information on the epidemiology of EHN in rainbow trout in south-eastern Australia was given by Whittington et al. 1999). The occurrence in rainbow trout appears to be confined to New South Wales at present (OIE, 2009). There have been no further peer-reviewed published reports on the occurrence of EHN in Australia but there have been several official reports of its endemic nature in the OIE Regional Aquatic Animal Disease Reports (Asia-Pacific region) for 2002-2009 but no details were given to allow assessment of their validity.

EHN has not been reported from any other country than Australia. Although very closely related ranaviruses (ECV and ESV) have caused heavy mortalities in catfish and sheatfish in Europe and once thought likely to be strains of EHNV, these are not now regarded as strains of EHNV by the International Committee for Taxonomy of Viruses or by OIE (2009). The Defra/OIE International Database on Aquatic Animal Diseases (www.collabcen.net) shows in the OIE data section that Belgium, Finland, Germany and Slovenia have reported the presence of EHN but it is almost certain that these are cases of ECV and/or ESV. Other countries reporting occurrence of EHN to OIE include India, Kuwait and Peru but without any supporting diagnostic or other evidence these reports were excluded from the review data entered into the EFSA database.

References

EFSA (2008). Aquatic species susceptible to diseases listed in Directive 2006/88/EC. The EFSA Journal, 808, 1-144

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OIE (2009). Manual of Diagnostic Tests for Aquatic Animals 2009. OIE, Paris, p222

Pierce, B.E., Phillips, P.H. & Jackson, G. (1991). Redfin virus. Fish disease confirmed in South Australia. SAFish, 15 (3), 5-6.

Whittington R.J., Kearns C., Hyatt A.D., Hengstberger S. & Rutzou T. (1996). Spread of epizootic haematopoietic necrosis virus (EHNV) in redfin perch (Perca fluviatilis) in southern Australia. Aust. Vet. J., 73, 112–114.

Whittington R.J., Philbey A., Reddacliff G.L. & Macgown A.R. (1994). Epidemiology of epizootic haematopoietic necrosis virus (EHNV) infection in farmed rainbow trout, Oncorhynchus mykiss (Walbaum): findings based on virus isolation, antigen capture ELISA and serology. J. Fish Dis., 17, 205–218.

Whittington R.J., Reddacliff L.A., Marsh I., Kearns C., Zupanovic Z. & Callinan R.B. (1999). Further observations on the epidemiology and spread of epizootic haematopoietic necrosis virus (EHNV) in farmed rainbow trout Oncorhynchus mykiss in southeastern Australia and a recommended sampling strategy for surveillance. Dis. Aquat. Org., 35, 125–130.

Table 1: list of host species

Redfin perch	*Perca fluviatilis*
Rainbow trout	*Oncorynchus mykiss*

Table 2: list of countries and locations

Australia	Victoria, New South Wales, Australian Capital Territory and South Australia

Epizootic Ulcerative Syndrome: disease detection, pathogen identification, and typing

Review author and date

Dr. Birgit Oidtmann
Cefas, Weymouth, United Kingdom

October 2009

Causative agent description

EUS is caused by the oomycete known as *Aphanomyces invadans* or *A. piscicida*, from here on referred to as *A. invadans*. Information on the agent, characteristics of the disease and the range of susceptible species has been reviewed in a number of recent publications or reports (Lilley et al., 2001b; Baldock et al., 2005; EFSA, 2007, 2008; World Organisation for Animal Health OIE, 2009).

So far only one genotype of *A. invadans* is recognised. Lilley et al. 1997 (Lilley et al., 1997a) compared 20 isolates of *A. invadans* from several geographic regions (Bangladesh, Thailand, Indonesia, the Philippines, Australia and Japan) by random amplification of polymorphic DNA (RAPD)-PCR using 14 10-mer primers and concluded that these isolates were not only conspecific (the adoption of the name Aphanomyces invadans was proposed), but probably constitute a single clonal genotype. Since then, an increasing number of sequences of *A. invadans* isolates have been made publicly available on genbank and other sources, confirming that there is very little sequence difference between the various isolates.

Diagnostic methods

A number of PCR methods have recently been published, with primers targeting the internal transcribed spacer (ITS) region or nearby located sequences of the pathogen (Lilley et al., 2003; Phadee et al., 2004a; Phadee et al., 2004b; Vandersea et al., 2006; Oidtmann et al., 2008). The ability to confirm the identity of isolates and detect the pathogen in clinical samples has greatly improved the capability of diagnosing the disease and detecting infection.

The ITS region is a target sequence frequently used for design of primers for specific diagnostic PCR assays. The ITS region has been shown to be prone to mutation, making it a suitable area in order to discriminate between closely related species. A potential disadvantage of targeting this area is that mutation may also occur over time within a given species, and therefore carrying the risk that primers do not detect strains with mutations in the target region. Sequences from about 20 *A. invadans* strains are publicly available for the ITS region or parts thereof. Small sequence differences amongst these strains can be found. Some of the sequence differences are in the target area of primers for *A. invadans* PCR assays. The potential consequence of such sequence differences is a potential loss in test sensitivity or even failure to detect. A comparison of 3 published PCR assays is provided in (Oidtmann et al., 2008).

Other methods for identification include isolation of the protist in pure culture, followed by an assessment of the morphology of hyphae, zoosporangia, type of zoospore release, macroscopical appearance in pure culture and growth characteristics at certain temperatures. However, due to the absence of sexual stages in its reproductive cycle, an important criterion for species determination is not available, allowing characterisation based on morphological criteria only to family level, and not species level.

Lilley published several papers in which he compared characteristics of several *A. invandans* strains from several geographical areas and found the various strains to be very consistent for

a broad range of features, such as band patterns in western blots, RAPD PCR, growth velocity and further more (Lilley et al., 1997a; Lilley and Inglis, 1997; Lilley and Roberts, 1997; Lilley et al., 1997b; Lilley and Supranee, 2000; Lilley et al., 2001a; Lilley et al., 2003).

Achieving agreement on the aetiological agent being *A. invadans* has taken several years. This was largely due to the difficulties in isolating *A. invadans* from infected fish (Baldock et al., 2005). This also means not all strains associated with outbreaks in new geographical regions have been isolated and were therefore not available for sequencing. Therefore, new strains may have evolved, but have not been identified.

Attempts should therefore be made to isolate the pathogen in each new outbreak, to ensure changes in the genome are being recognised.

Gaps in knowledge

There are a number of areas where information is still lacking:

1. It is unclear how A. invadans survives during periods where no noticeable mortalities are observed. There might be a carrier status in fish, which yet remains to be detected.
2. The susceptible species range is likely to grow further. Each time the pathogen reaches a new geographical area, usually a large range of species are found to be susceptible. Therefore, it can be expected that further fish species will be identified as susceptible to infection.
3. Susceptibility studies in new species are usually conducted by injecting zoospores into fish muscle tissue. Simple bath challenges have failed in the majority of species tested with the only species in which infection was successfully achieved in bath challenges being Atlantic Menhaden. Challenge experiments with strains of A. invadans from Southeast Asia have suggested that zoospores attach to the dermis and not the epidermis before invasion (Lilley et al., 1998). Skin injury is, therefore, considered to be required to provide a portal for infection. However, it remains to be investigated whether prior skin damage is always present in epizootics of the disease or whether other conditions may weaken the skin barrier.

References

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Phadee, P., Kurata, O., Hatai, K., 2004a. A PCR method for the detection of Aphanomyces piscicida. FISH PATHOL 39, 25-31.

Phadee, P., Kurata, O., Hatai, K., Hirono, I., Aoki, T., 2004b. Detection and Identification of Fish-Pathogenic Aphanomyces piscicida Using Polymerase Chain Reaction (PCR) with Species-Specific Primers. Journal of Aquatic Animal Health 16, 220-230.

Vandersea, M.W., Litaker, R., Yonnish, B., Sosa, E., Landsberg, J.H., Pullinger, C., Moon-Butzin, P., Green, J., Morris, J.A., Kator, H., Noga, E.J., Tester, P.A., 2006. Molecular Assays for Detecting Aphanomyces invadans in Ulcerative Mycotic Fish Lesions. Applied and Environmental Microbiology 72, 1551-1557.

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Epizootic Ulcerative Syndrome: occurrence and distribution

Review author and date:

Dr. Birgit Oidtmann
Cefas, Weymouth, United Kingdom

October 2009

Selection of studies

ASFA and Scopus databases were searched for references using the search terms *Aphanomyces invadans*, *Aphanomyces invaderis*, *Aphanomyces piscicida*, and Epizootic Ulcerative Syndrome.
However, a large number of publications were sources referred to in other published papers, through email alerts, (aquavetmed, David Scarfe), or were in the collection of publications of the reviewer, which had been built up over the last few years.

Due to the fact that molecular tools designed to specifically detect *A. invadans* have only become available fairly recently, most of the earlier publications on Epizootic Ulcerative Syndrome (EUS) (or mycotic granulomatosis (MG), red spot disease (RSD) and ulcerative mycosis (UM)) did not provide a full confirmation of the identity of the fungal pathogen found in affected fish. Similarly, the primary cause of EUS was debated for a long time. Some authors followed other hypothesis rather than the fungal pathogen one and may not have reported any evidence pointing towards an involvement of a fungal pathogen. Therefore, the presence of fungal hyphae or granulomatous mycosis as a histopathological feature observed in affected fish may have been overlooked or not reported. If another publication was available for a country, which did provide a description of mycotic granulomatosis or confirmation of the identity of the isolated fungal cultures as Aphanomyces invadans, these were preferably chosen for inclusion in the data table.

Data and analyses

Around 300 references were considered for the review. Twenty-seven publications were included in the data table. Publications for inclusion into the data table were in the first instance selected on the basis that they were reporting EUS in a new country and that the description of the disease was providing at least evidence of mycotic granulomatosis based on histopathology. Several reports describing such histopathological changes may have been available for a certain country. However, it was not attempted to compile a comprehensive list of every publication, which reported mycotic granulomatosis in fish for a respective country.

Studies were also included, if the publication reported the source country of affected fish and the identity of *A. invadans* had been confirmed using species specific molecular diagnostic methods. If neither nor were available for a country, for which EUS had been reported, it was resorted to publications that mentioned the presence of the disease without the supporting evidence of histopathology or molecular methods.

The quality ratings applied to the evidence presented for occurrence were:

- **Definite:** was only given, if details required for full identification of the pathogen and typical histopathological changes were reported were available ((a) histopathological findings consistent with Aphanomyces invadans infection (granulomatous mycosis in known target tissues, such as muscle of skin); (b) confirmation of A. invadans by PCR / through sequencing).

- **Probable:** was given if either the identity of the pathogen was established as A. invadans (or A. piscicida), or if the histopathological findings were consistent with EUS. On some occasions (e.g. FAO report 2009), the rating probable was also given if the listing was based on information provided by the OIE reference laboratory for EUS.
- **Possible:** a number of publications list several species as being susceptible to EUS. The criteria for listing a species are not provided by the authors. In the same publication, data may have been provided mentioning that mycotic granulomatosis was sometimes observed, without specifically identifying the species in which this histopathology was found.
- **Doubtful:** diagnostic procedures not provided.

Summary of disease findings

EUS appears to have first occurred in Japan in 1971 in Ayu (*Plecoglossus altivelis*) (Egusa and Masuda, 1971) followed by a report of the disease in Australia in 1972 (McKenzie and Hall, 1976). Since then, the disease has been repeatedly confirmed in various species for Japan and Australia and has spread further across several Asian countries. An ulcerative mycosis was observed in estuarine fish along the east coast of the United States since 1978 (and perhaps before) (Noga and Dykstra, 1986) and since then the disease has been repeatedly reported from the USA in a wide range of species. EUS was for the first time found on the African continent in 2006 (Andrew et al., 2008; FAO, 2009). No reports are yet published showing the presence of the disease in Europe, or South America.

Supported by probable or definite reports, EUS has occurred in the following countries:
Africa: Namibia, Botswana, Zambia; Asia: Bangladesh, Burma (Myamar), India, Indonesia, Japan, Laos People's Democratic Republic, Malaysia, Nepal, Philippines, Sri Lanka, Thailand; Australia and USA.

The available publications only provided evidence of the possible presence of EUS for Papua New Guinea, Vietnam, Nepal and Pakistan (Vietnam, Nepal and Pakistan reported in (Lilley et al., 2001) as: Regional scientists working with AAHRI staff have succeeded in histologically demonstrating EUS in areas that were previously unconfirmed, e.g. Vietnam (Phan Thi Van, pers. comm.), Nepal (S. Dahal, pers. comm.) and Pakistan (R. Anjum, pers. comm.)).

The evidence for the presence of EUS in Singapore is indirectly provided through a publication, describing the disease in ornamental fish imported from Singapore and sampled from aquarium shops in Japan. The presence of EUS in Cambodia is unconfirmed (Lilley et al., 2001, page 23).

In addition to the reviewed publications, the OIE WAHID website was queried regarding reporting of EUS worldwide. The data on the OIE WAHID website only cover the time period from 2005 until now. The following information for countries not mentioned elsewhere, or with only possible evidence was obtained:
Iraq: suspicion reported to OIE 2007 and 2008, but unconfirmed
Nepal: suspicion reported to OIE in 2006 and 2007, but unconfirmed

A wide range of fish species, including both marine / estuarine and freshwater species, has been found to be susceptible to infection with Aphanomyces invadans. A few species appear have been reported as being “resistant”; these include Tilapia (Vishwanath et al. 1997b), Stickleback (Khan et al 1998), Oreochromis mossambicus (Coates et al. 1989 or Coates 1984, cited in Roberts et al. 1994), Carp, Eel, Loach, catfish (Hatai 1980, cited in Baldock et al. 2005), Chinese carp and European carp (Roberts et al. 1989). However, there are also publications reporting susceptibility in some of these species. Therefore, reports of apparently resistant species need to be interpreted carefully.

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Table 1: list of host species

See text summary.

Table 2: list of countries and locations

Australia	lower Clarance River, north-eastern New South Wales; lower Richmond River, north-eastern New South Wales; New South Wales; Northern Queensland; Queensland; Northern Territories; Saltwater Creek,
Bangladesh	Mymensingh; Parbatipur; Srimanagal
Botswana	Chobe River; Kasane
India	Karnataka
Indonesia	
Japan	Chiba Prefecture; Oita; Miyazaki; Tokyo (fish imported from Singapore); Tokushima; Shiga; Nagano; Tochigi Prefectures; Lake Kasumigaura, Ibaragi Prefecture; Kyushu Island; Kojima Bay, Okayama Prefecture;
Laos PDR	
Malaysia	
Myanmar (Burma)	
Namibia	
Philippines	North Luzon; Central Luzon; South Luzon; Coastal lagoon, Buguey

	(Cagayan Province Northern Luzon); Buguey (Cagayan Province, Northern Luzon); Bautista (Pangasinan Province, Central Luzon); Pulinan (Bulacan Province, Central Luzon); Laguna Lake (Laguna Province, Southern Luzon)
Sri Lanka	Bellanwila-Attidiya wetland; near Bellanwilla temple; main branch of Bolgoda Canal; 15 km northeast of Colombo (spread from there northward spread up to Chilaw and southwards to Kalutara)
Thailand	Bangkok (experimental trials)
USA	Florida; North Carolina
Zambia	

Infectious haematopoietic necrosis (IHN): disease detection, pathogen identification and typing

Review author(s) & Date:

Dr Peter Dixon,
Cefas, Weymouth, United Kingdom

October 2009

Causative agent description

The causative agent is IHN virus (IHNV), Family *Rhabdoviridae*, Genus *Novirhabdovirus*. Key information on the virus and disease can be found in recent publications (Bergmann and Fichtner, 2008; OIE, 2009). There appears to be one serotype in comparisons using polyclonal antisera, although sub-types/variants have been reported using monoclonal antobodies. Different electropherotypes have been described, but the method currently most widely used for strain differentiation is the reverse transcription polymerase chain reaction (RT-PCR) (e.g. Kurath et al., 2003).

Summary of methods findings

The method recommended (OIE, 2009) for surveillance for the virus is isolation in cell culture followed by identification using a serological or molecular biology method. Methods recommended (OIE, 2009) for diagnosis of clinical disease are isolation in cell culture followed by identification using a serological or molecular biology method, or any two of the following tests: antibody-based assays, DNA probe or RT-PCR. RT-PCR should always be followed by sequencing to confirm the identity of the amplicon.

However, molecular methods of virus identification have only been used in relatively recent times and many of the sources in the datatable were published before then. In such cases identification of the virus usually relied on serological methods. Those identifications are reliable as long as validated antisera and appropriate controls were used, but that is not always apparent from the publications.

There are a number of primer sets published for the identification of IHNV, and recommended primers are now published in the OIE manual. However, some studies have used new primer sets that have not been compared with existing primer sets, and so there is no information on the comparative sensitivity of the primers used.

Gaps in knowledge

See above for comments on sensitivity/specificity of primer sets used for RT-PCR.

References

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Infectious haematopoietic necrosis (IHN): occurrence and distribution

Review author(s) & Date

Dr Peter Dixon
Cefas, Weymouth, United Kingdom

October 2009

Selection of studies

ASFA and Scopus were databases most frequently used. Search terms included: IHN*, infectious haematopoietic necrosis. Databases were searched during 2009. However most of the documents assessed were in the Cefas library system and were electronically or manually searched.

Data and analyses

Over 600 documents were appraised, and data from 27 entered into the data table. The aim was to include studies reporting the isolation/detection of the virus, particularly if they reported a new country or new host species. Papers recording the detection of the virus based on reverse transcription polymerase chain reaction alone were in general not included, and definitely not if sequencing was not done. Some of the studies appraised collected a number of isolate from different sources and then compared them by serological or molecular biology methods. Most often the only information about an isolate was host species, location and year of isolation although all three attributes may not have been reported. Such studies may not have been included unless they added a new host or country, or added data to another publication already in the data table.

The quality ratings applied to the evidence presented for occurrence were:

- **Definite**: full details of identification methods were given or confirmation came in a subsequent paper or confirmation was done in a second laboratory; validated methods used.
- **Probable**: Most details of identification were given; validated methods used.
- **Possible**: Some details of identification given; no confirmation reported.
- **Doubtful**: Scant or no details given and or non validated methods used; no confirmation reported.

Summary of disease findings:

The disease was initially identified in the USA but the virus has been reported to be present in 18 other countries or regions including many European countries: Austria, Belgium, Canada, Chile, China, Croatia, Czech Republic, France, Germany, Iran, Italy, Japan, Korea, Russia, Slovenia, Spain, Switzerland and Taiwan. The hosts are predominantly salmonids, particularly Pacific salmon. The virus had been reported from a small number of non-salmonids from both marine and freshwater environments although the confirmatory data have sometimes been lacking.

Mortalities and importance:

The disease has a major effect on salmonid aquaculture, and a licensed vaccine is available in North America. A number of countries undertake surveillance for the virus and many of the initial isolations of virus in new hosts or countries have been as a result of surveillance

programmes. In such instances, there may be no information on disease occurrence in either the species or the country. From the publications there is often no information on the relevance of the findings reported. However, this may not always be known until some time after the original report and it may then be difficult to have a follow-up report published.

Other comments:

Appraisal of the documents often revealed the lack of information about the circumstances of the isolation (field data) and about the actual isolation and identification of the virus (laboratory data). One outcome of this project could be a proposal for a standardised reporting system for disease outbreaks and surveillance studies.

Infectious salmon anaemia (ISA): disease detection, pathogen identification and typing

Review author(s) & Date:

Richard Paley
Cefas, Weymouth, United Kingdom

October 2009

Causative agent description:

Infectious salmon anaemia is a disease of seawater farmed Atlantic salmon (*Salmo salar*) infected with Infectious salmon anaemia virus (ISAV), the type species virus of the newly classified genus *Isavirus* within the family *Orthomyxoviridae*. ISAV virions are enveloped, 100-130 nm in diameter with a single stranded, negative polarity RNA genome in eight segments. This genome encodes at least ten proteins including viral polymerases PB1, PB2 and PA (segments 1, 2 and 4); nucleoprotein, matrix protein (segments 3, 8-ORF1), glycoproteins haemagglutinin-esterase (HE) and fusion protein, (segments 6 and 5); non structural interferon antagonist (7-ORF 1); RNA-binding, structural interferon antagonist (8-ORF 2) and a potential nuclear export protein (7-ORF 2). A further third ORF from segment 7 has been proposed. (See OIE 2009 for relevant references to all the above).

Initial studies of ISAV genome sequence from segments 2 and 8 and revealed clear strain differences grouping European and North American isolates separately (Blake *et al*., 1999; Inglis et al., 2000) but these segments are relatively well conserved and less informative within European isolates (Krossoy *et al*., 2001). Based on subsequent sequencing of segments 6 and 5 the European group can be further divided into 3 subgroups (Devold *et al*., 2006). A highly polymorphic region (HPR) was identified in the HE gene and deletions in this region are associated with virulence whilst RT-PCR and sequencing has identified a presumed low or avirulent strain of ISAV possessing a full length HPR (HPR0) circulating in fish in the wild (Markussen *et al*., 2008). A further virulence marker has been identified in the fusion protein (Markussen et al., 2008).

Summary of methods findings:

The methods recommended for surveillance (OIE 2009) are isolation in cell culture (followed by virus identification) or RT-PCR or real-time RT-PCR. SHK-1, ASK, TO or CHSE-214 cell lines are suitable susceptible cell lines. CPE may not always be evident so all cell cultures should be subsequently tested by a confirmatory method. The methods recommended for confirmation are IFAT on kidney imprints and cell cultures, Immunohistochemistry, RT-PCR or real-time RT-PCR. Antibody based detection methods are specific but not as sensitive as virus isolation, or PCR based methods hence not recommended for surveillance. Care should be taken when using RT-PCR on cell culture as this cannot differentiate replicating virus in culture from carryover virus from the initial inoculum unless quantification by real time RT-PCR is applied. Sequencing is required for genotyping. Primers designed in the more conserved segments 2 and 8 are recommended for diagnostics as they allow detection of all known strains isolated to date whilst segments encoding the glycoprotein genes (Segments 5 and 6) are recommended for epidemiological tracing (OIE 2009).

Gaps in knowledge

Sequence data is not available for many of the early outbreaks in Norway. This combined with the fact that the HPR0 circulating low/avirulent strain cannot be grown in culture means that there is limited information on the triggers for or frequency of development of virulent strains.

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OIE (2009) Manual of Diagnostic Tests for Aquatic Animals 2009. OIE, Paris, p222

Infectious salmon anaemia: occurrence and distribution

Review author(s) & Date:

Richard Paley
Cefas, Weymouth, Kingdom

October 2009

Selection of studies:

PubMed, Scopus, Promed and WAHID databases were searched with the terms “infectious salmon ana(e)mia”, ISA and ISAV in June and July 2009 and updated in October 2009. 137,154, 24 and 30 references were returned respectively. Google searches were performed for “infectious salmon ana(e)mia” and “Faroes” and “Ireland” and “Norway” to obtain non peer reviewed reports, a specific request for information was directed to the Marine Institute, Ireland. Items relevant to disease outbreaks, host species, experimental transmission, pathogen characterisation and diagnostic methodology were retained. In the main only references pertaining to disease outbreaks in new locations or new host species were included in the data table. Quality ratings were assessed on the basis of methods of identification and in particular sequence analysis.

Data and analyses:

Over 200 source documents were reviewed and those retained are listed in the provided Reference manager database (ISAV2) with extracted data from 15 tabulated in [isa-cefas-final].

The quality ratings applied to the evidence presented for occurrence were:

- **Definite (or good)**: full details of identification methods were given or confirmation came in a subsequent paper or confirmation was done in a second laboratory; validated methods used (ISA specific mAb or RT-PCR and sequencing).
- **Probable**: Most details of identification were given; validated methods used, report may not have been published in peer reviewed journal but from respected source.
- **Possible**: Some details of identification given; no confirmation reported.
- **Doubtful**: Scant or no details given and or non validated methods used; no confirmation reported.

Summary of disease findings:

Essentially a disease of farmed Atlantic salmon in seawater, the first recorded mortalities due to ISA were in Norway in 1984. It was a further 11 years before the causative agent was identified. The disease rapidly spread throughout salmon farms along the coast of Norway and by 1991 incidence peaked with 80 recorded outbreaks. A control programme introduced in Norway dramatically reduced the number of outbreaks with between one and twenty reported cases per year from 1994 to date. Initially reported as Haemorrhagic Kidney Syndrome, ISA was reported in New Brunswick, Canada in 1996 and subsequently just over the border in Maine, USA in 2000. Efforts towards a harmonized control program including regular surveillance has reduced but not eliminated the incidence of disease in the region (Gustafson *et al*., 2008). There have been two outbreaks in the UK, one on the West coast of Scotland from 1998 to 1999 with 11 confirmed cases and a second in the Shetland Islands in

2009 with 5 confirmed cases currently under eradication programme. ISA was reported from numerous sites in the Faroe Islands between 2000 and 2005. Chile reported outbreaks in Atlantic salmon in 2007 which have spread rapidly within regions X, XI and XII such that the disease is currently classified as endemic in Chile (Maradones *et al*., 2009). ISA has been isolated from diseased Coho salmon in Chile. ISAV was isolated in cell culture from Rainbow trout in Ireland during surveillance in 2002 (unpublished, Fiona Geoghegan pers. comm.) but there were no signs of disease nor associated mortalities. There are some reported cases of isolation from salmon in freshwater (Vike *et al*., 2009). ISA has been detected in brown and sea trout by RT-PCR and virus has been isolated from sea trout. Virus has been detected by RT-PCR in kidney material from experimentally infected herring (for references other than those given see OIE 2009).

Mortalities and importance:

ISA is an extremely significant disease in countries with large salmon farming industries, mortality is often severe (upto 80%). 1999 annual production losses due to ISA were estimated at US$ 11M and 14M in Norway and Canada respectively whilst the UK 1998-99 outbreak was estimated to cost US$32M (Hastings *et al*., 1999). Salmon production in Chile has declined by over 50% in the two years since virus discovery in 2007 and up to 20000 workers have been made redundant as a result. An estimated $1b is required to recover lost biomass (Carvajal, 2009).

Other comments:

Demonstration of true vertical transmission remains a contested issue. Vaccination is undertaken in USA, Canada, the Faroe Islands and Chile.

Table 1: list of host species

Atlantic salmon	*Salmo salar*
Sea trout, brown trout	*Salmo trutta*
Coho salmon	*Oncorhyncus kisutch*
Rainbow trout	*Oncorhynchus mykiss*
Atlantic herring	*Clupea harengus*

Table 2: countries and locations

Country	**Location descriptor**
Norway	Widespread throughout the coast of Norway since 1984
Canada	New Brunswick, Bay of Fundy, repeated isolations since 1996
UK	Scotland, One outbreak from 1998-99 on the West Coast and one current (2009) in the Shetland Islands
Faroe Islands	Widespread outbreaks between 2000 to 2005
USA	Maine, Cobscook Bay, repeated isolations since 2000
Ireland *	Virus isolated by cell culture from Rainbow trout during surveillance in 2002 but disease and mortality was not evident.
Chile	Widespread in regions X, XI and XII since 2007

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Vike S, Nylund S, Nylund A (2009) ISA virus in Chile: evidence of vertical transmission. Arch.Virol. 154:1-8

Koi herpesvirus disease: disease detection, pathogen identification and typing

Review author(s) & Date:

Keith Way
Cefas, Weymouth, United Kingdom.

Olga Haenen, Central Veterinary Institute of University of Wageningen, AB Lelystad, Netherlands.

October 2009.

Causative agent description:

Characteristics of causative agent

Koi herpesvirus disease (KHVD) is a herpesvirus infection capable of inducing a contagious and acute viraemia in common carp (Cyprinus carpio) and varieties such as koi carp and ghost carp (OIE 2009). The causative agent is koi herpesvirus (KHV) although it has also been given the name carp interstitial nephritis and gill necrosis virus (CNGV) (OIE 2009). Waltzek *et al.* (2005) provided evidence to support the classification of the virus as a herpesvirus, and named it cyprinid herpesvirus 3 (CyHV3). The ICTV has accepted this nomenclature and updated the taxonomy of herpesviruses and placed CyHV3 as a species in the genus Ictalurivirus in the family Alloherpesviridae (Davison et al.2009).

Evidence for strain differentiation

The complete genome sequences of three KHV (CyHV3) strains isolated from Japan, Israel and the USA have been compared and the genomes were found to be highly similar to each other at the sequence level. Alignment of the three genome sequences identified some small variations that suggested that two lineages had arisen (Japan (J) & USA-Israel (U/I)) from a wild-type parent (Aoki et al 2007). An analysis of genomic regions based on PCR detection targets has confirmed a clear genetic distinction between Asian and European (including U/I) genotypes of KHV (Kurita et al. 2009). The analysis showed that Asian KHVs were very homogenous in contrast to European and U/I KHVs, where seven variants were identified. More recently a duplex PCR assay has been used to distinguish CyHV3-J genotype from CyHV3-U/I and avoid DNA sequencing. Among 42 samples from KHV-infected carp a third genotype, intermediate between the J and U/I genotypes, was identified (Bigarre et al. 2009).

Summary of methods findings:

The recommended method for presumptive diagnosis of KHV, for reasons of availability, utility and diagnostic specificity and sensitivity, is PCR. Other methods, such as presence of gross clinical signs, histopathology, transmission electron microscopy and isolation in cell culture, have application in some situations. However, cost, accuracy and other limitations may seriously limit their application (OIE 2009). For confirmatory diagnosis, PCR and PCR amplicon sequencing are recommended.

Two published single-round-PCR methods are currently considered to be the most sensitive for detection of KHV DNA in fresh tissue samples from clinically diseased carp (OIE 2009). The first uses the TK primer set developed by Bercovier et. al. (2005) and the second was developed by Yuasa et.al. (2005) and is an improvement of a published protocol developed by Gray et al. (2002). The OIE manual (2009) also states that if the tissue shows evidence of decomposition then primer sets targeting shorter regions of the genome may need to be used such as those developed by Hutoran et al. (2005). The manual also suggests that existing published primer sets can be modified to target shorter sequences of the KHV genome.

Gaps in knowledge

The Bercovier and Yuasa protocols may allow detection of sub-clinical levels of virus but more sensitive PCR methods are usually required to detect the low-levels of virus found in covertly infected carp. The more sensitive methods include real-time (qPCR) and nested PCR. In international PCR methods ring-trials, in 2007 and 2008, the more sensitive nested PCR and real-time PCR assays most reliably identified KHV in trial samples containing the lowest genome copy numbers (Way et al. 2009). The assay most commonly used in laboratories around the world is the Taqman real-time PCR method developed by Gilad et.al. (2004). Real time PCR assays target shorter regions of the genome and are also suitable for detecting the shorter viral DNA fragments found in decomposing tissue.

Some KHV variants have been detected during KHV disease outbreaks in the UK and also from healthy koi carp imported into the UK and The Netherlands (D. Stone & M. Engelsma, pers.comm.). These variants were not detected by the routine PCR methods. Variant DNA can be successfully amplified using generic cyprinid herpesvirus polymerase primers and the amplicons show a 95-98% similarity to the published KHV sequence for the DNA polymerase gene. Further studies are needed to determine the prevalence of these variants and their significance for disease outbreaks.

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Koi herpesvirus disease: occurrence and distribution

Review author(s) & Date:

Keith Way
Cefas, Weymouth, United Kingdom.

Olga Haenen
CVI of University of Wageningen, AB Lelystad, Netherlands

October 2009.

Selection of studies:

Sources accessed included Web of Science, OVID-medline, Current Contents, Promed, OIE website and WAHID interface, EU CRL Fish Disease Website and the extensive collections of publications held in the two laboratories. Searched during August 2009.

Search terms included KHV, KHVD, Cyprinid herpesvirus 3, koi, carp, herpesvirus and individual country names.

Data and analyses:

49 source documents were located and are listed in attachment [KHV - geo-papers OH-KW-01-Sep-09.doc] with extracted data tabulated in [Entrysheet-KHV OH-KW 01-Sep -2009.xls].

The quality ratings applied to the evidence presented for occurrence were:

- **Definite**: full details of identification methods were given or confirmation came in a subsequent study; validated methods used. Convincing evidence.
- **Probable**: Most details of identification were given; validated methods used but firm confirmation not achieved. Look very likely but some uncertainties
- **Possible**: Some details of identification given; no confirmation reported.
- **Doubtful**: Scant or no details given and or non validated methods used; no confirmation reported.

Summary of disease findings:

There are very few host species for the disease. Published reports describe KHV outbreaks in populations of common, koi and ghost carp (all *Cyprinus carpio*) and also KHV detection in goldfish populations. The review authors are aware of unpublished reports of detection of KHV in other cyprinid fish and some non-cyprinid fish species.

Since the first major outbreaks were reported in Israel in 1998, KHV disease has spread to most of the major continents in the world. The **KHV entrysheet** lists 12 countries in Europe, 8 in Asia, 2 in North America and one in Africa (Table 1).

Some reports have described disease outbreaks with clinical signs very similar to KHVD but the virus isolated is not KHV. It is possible that these carp populations have been suffering from KHVD but, for unknown reasons, KHV has not been isolated or detected.

Mortalities and importance:

KHVD has caused mass mortalities in carp populations and has a major impact on ornamental carp and edible carp production and is a serious threat to wild carp populations.

This has led to the disease being listed by the OIE and the EU, and is also listed in many countries at national level.

Possible sources of bias:

The review authors are aware, from personal communications and presentations at meetings, of un-reported KHVD outbreaks or KHV detections in other countries. These outbreaks and detections have occurred in koi carp populations in closed and isolated ornamental fish import facilities. There is the possibility that the virus has spread to native carp populations through release of diseased koi carp but no KHVD outbreaks in natural carp populations have been reported from these countries. KHVD was not listed by the OIE until 2007 and many KHVD outbreaks that occurred before 2007 were not notified to OIE, particularly if the disease was only seen in koi carp. There is concern that the occurrence of KHVD is not always notified to the Veterinary Authorities in a country and consequently is not notified to the OIE.

There are reports of extensive KHVD outbreaks in Indonesia and reports of isolated outbreaks in Malaysia and Thailand. In these Asian countries the water temperatures in natural waters are only permissive for virus infection during limited periods of the year. There are concerns that there may have been an inapparent spread of KHV to other countries in that region of Asia through movements of koi carp.

Other comments:

None

Table 1: Countries and locations

Country	Location descriptor
Austria	Not specified
Belgium	1 site in Hainaut, (Familleureux), 1site in Namur (Oheye)
Canada	2 or more lakes in Ontario (wild carp)
Czech Republic	Not specified. Survey of koi and common carp farms in 2005 and 2006
Denmark	4 sites, all koi import sites.
France	8 isolations reported from koi, probably representing 7 different sites.
Germany	Saxony (3 sites) and North-Thuringia (1 site): 10 sites elsewhere in Germany. First detection in koi in 1997 and in farmed common carp in 2000.
Indonesia	East-Java: Blitar province than spread to Bandung; Sumatra and West Papua (Sentani Lake); West-Java: Subang Regency, Saguling and Cirata reservoirs in the Citarum river system; South-Sumatra: Lubuk Lingau Regency
Israel	Carp farms along coastal region; North (Mishor-Hahof region and Jordan valley); Northern to Southern Central Israel: 90% of all carp farms in Israel affected.
Japan	Kanagawa prefecture; Kanagawa & Saitama Prefecture; Gunma prefecture; Kinki District (Kyoto Prefecture); Ibaraki prefecture (Lake Kasumigaura& Kitaura); Okayama prefecture.
Korea (Republic of)	Confirmed in Gangwon; Suspicion in Kangwon Province, Kyunggi Province, Chungbuk Province, Chunnam Province & Kyungbuk Province.
Malaysia	Peninsular Malaysia: 1 koi export site KHV positive.
Poland	Dolna Odra power station: One area but more than one site (farmed carps) and 4 carp farms elsewhere in Poland.
Serbia	Not specified
Singapore	1 import site, ornamental koi
Slovenia	Maribor and Ptuj in farmed carp
South Africa	Outbreaks in 2004 in Western Cape, Gouteng and KwaZulu Natal, koi/carp not specified, 3 sites.
Sweden	Skane Lan (Kristianstad) Ornamental koi.
Switzerland	Not specified
Taiwan	Northern Taiwan, ornamental koi.
Thailand	Chiang Mai province (Mae Jo) 1 site.
The Netherlands	Ornamental koi, at least 15 sites and 3 common carp sites.
United Kingdom	England & Wales: 23 cases of KHV in wild carp in 2006 and 39 cases from 2003 to 2006. First confirmed outbreaks in koi in 2000.
USA	California; New York state ; Arizona ; Texas; South Carolina; several outbreaks at ornamental koi sites but also in wild carp populations.

Viral haemorrhagic septicaemia: disease detection, pathogen identification and typing

Review author(s) & Date:

Dr Peter Dixon
Cefas, Weymouth, United Kingdom

October 2009

Causative agent description:

The causative agent is VHS virus (VHSV), Family *Rhabdoviridae*, Genus *Novirhabdovirus*. Key information on the virus and disease can be found in recent publications (Bergmann and Fichtner, 2008; OIE, 2009). There are three serotypes of VHSV: I, II and III based on neutralisation with polyclonal antisera. However, using neutralising monoclonal (MAb) and polyclonal (PAb) antisera, VHSV isolates could also be divided into three serotypes, but they differed in members from the original three serotypes (Olesen et al., 1993). Subsequently isolates not conforming to those neutralisation patterns have been identified using MAbs, and different alignments of isolates can be made using different panels of MAbs. Now the isolates are more usually compared and grouped according to their full or partial gene sequences. Different genogroupings have been proposed and these have been discussed by Dr David Stone in an accompanying document.

Summary of methods findings:

The method recommended (OIE, 2009) for surveillance for the virus is isolation in cell culture followed by identification using a serological or molecular biology method. Methods recommended (OIE, 2009) for diagnosis of clinical disease are isolation in cell culture followed by identification using a serological method or reverse transcription polymerase chain reaction (RT-PCR) followed by sequencing of the amplicon. A suspect case of VHS (as defined in the relevant OIE chapter) can also be confirmed by detection of the virus in tissues or tissue preparations by immunoassay using specific anti-VHSV antibodies or detection of the virus by RT-PCR followed by sequencing.

However, molecular methods of virus identification have only been used in relatively recent times and many of the sources in the datatable were published before then. In such cases identification of the virus usually relied on serological methods. Mostly the analyses were done with PAbs that allowed separation of an isolate into one of the three original serotypes. Often the same PAbs were used in different laboratories, but sometimes “in house” antisera were used that had not been fully compared with other PAbs. As the titre of many of the PAbs was relatively low, they were often used at low dilution, which could have resulted in non-specific neutralisation. Hence, some isolates may have been misallocated to a serotype.

The use of molecular biology methods for the identification of VHSV has been discussed by Dr David Stone in an accompanying document.

Gaps in knowledge

See accompanying document by Dr David Stone.
A number of publications examined report the detection of a virus or viruses, but the viruses are not typed. Sometimes this is done later in a subsequent publication. It would be very useful if the typing was reported in the initial publication.

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Viral haemorrhagic septicaemia: occurrence and distribution

Review author(s) & Date:

Dr Peter Dixon
Cefas, Weymouth, United Kingdom

October 2009

Selection of studies:

ASFA and Scopus were databases most frequently used. Search terms included: VHS*, viral h?emorrhagic septic?emia. Databases were searched during 2009. However most of the documents assessed were in the Cefas library system and were electronically or manually searched.

Many of the reports of VHSV in species in the Great Lakes area of North America are in "grey" literature, but often the source is a Government Agency. In such cases, the reports have been taken as "Definite" as it is known that the Agency would not list a species as being susceptible without stringent laboratory work in support of the report.

Data and analyses:

Nearly 700 documents were appraised, and data from 70 entered into the data table. The aim was to include studies reporting the isolation/detection of the virus, particularly if they reported a new country or new host species. Papers recording the detection of the virus based on PCR alone were in general not included, and definitely not if sequencing was not done. Some of the studies appraised collected a number of isolate from different sources and then compared them by serological or molecular biology methods. Most often the only information about an isolate was host species, location and year of isolation although all three attributes may not have been reported. Such studies may not have been included unless they added a new host or country, or added data to another publication already in the data table.

The quality ratings applied to the evidence presented for occurrence were:

- **Definite**: full details of identification methods were given or confirmation came in a subsequent paper or confirmation was done in a second laboratory; validated methods used.
- **Probable**: Most details of identification were given; validated methods used.
- **Possible**: Some details of identification given; no confirmation reported.
- **Doubtful**: Scant or no details given and or non validated methods used; no confirmation reported.

Summary of disease findings:

The disease originally occurred in rainbow trout in freshwater in a number of European countries, but the virus has subsequently been identified in salmonid and non-salmonid fish in freshwater and sea water in many parts of the world. The virus has been associated with disease in both freshwater and marine species, but experimental infection trials have shown that whilst freshwater isolates may produce disease in marine fish, marine isolates, in general, have no or low virulence for freshwater species. The majority of isolations from marine fish have been during surveys and there is no information on whether the virus causes disease in those species. In European countries the virus predominantly occurs in salmonid

fish in freshwater, and when it has been found in non-salmonid fish they have generally been cultured or ranched. Recently, however, in the Great Lakes area of North America, VHSV of a new genotype has been isolated from a large number of non-salmonid wild freshwater fish.

Mortalities and importance:

The disease is of great economic importance to aquaculture, and development of an effective vaccine has been a goal for decades. A vaccine against VHSV has been licensed for use in Germany, but no vaccine has been licensed for use in any other country.

Other comments:

Appraisal of the documents often revealed the lack of information about the circumstances of the isolation (field data) and about the actual isolation and identification of the virus (laboratory data). One outcome of this project could be a proposal for a standardised reporting system for disease outbreaks and surveillance studies.

Table 1: Countries and locations

Country	Location descriptor
Austria	
Baltic Sea	
Belgium	
Black Sea	
Bulgaria	
Canada	Coast of British Columbia
Czech Republic	
Denmark	
English Channel	
Finland	
France	
FRG	Baltic coast of FRG
Germany	
Iran	
Ireland	
Italy	
Japan	Coastal waters
Kattegat	
N Atlantic	W of Sula; Kildas; NE Rona; W Barra; W of Orkneys; Flemish cap
North Pacific	North Pacific
North Sea	East Bank; Bergen Ground, Balta, Bergen Bank, Beryl; Patch
Norway	
Poland	
Romania	
Skaggerak	Skaggerak; Skaggerak/North Sea

Slovakia	
Spain	
Spain or Newfoundland	(lab study comparing genotypes)
Sweden	
Switzerland	
United Kingdom	
USA	Auke Bay, Alaska; Kodiak Island, Alaska; Lisianski Inlet, Alaska; Pacific coast of California; Pacific coast of Oregon; Prince William Sound, Alaska; Puget Sound, Washington;
USA/Canada	

2) Mollusc diseases

Infection with *Bonamia exitiosa*: disease detection, pathogen identification and typing

Review author(s) & Date:

Marc Engelsma
CVI of Wageningen UR, Lelystad, Netherlands

Mike Hine
Cefas, Weymouth, UK

October 2009

Causative agent description:

Characteristics of causative agent
Bonamia exitiosa is an obligate intra-haemocytic protozoan parasite of oysters of the family Ostreidae. It is phagocytosed by the oyster haemocytes in which it forms a parasitophorous vacuole, feeds and divides until the progeny burst from the remains of the haemocyte to infect other haemocytes. It uses lipid vesicles from cytoplasmic lipid droplets to alter the phagosome membrane and form the parasitophorous vacuole, and hydrolytic enzymes in the lipid droplets to digest the host cell cytoplasm. *Bonamia exitiosa* causes serious disease in wild stocks of *Ostrea chilensis* in New Zealand and *Ostrea angasi* in Australia, at low to moderate temperatures and full salinity. The parasite causes very different histopathology in these two hosts. In *O. angasi* it occurs in focal gill lesions, thought to be the point of entry, and low numbers of parasites are observed in large focal necrotic lesions. The parasite is epitheliotropic. In *O. chilensis* in New Zealand infections are disseminated throughout the oyster, there are no gill lesions, although congested gills may burst. Large numbers of parasites may be present, and the parasite is not epitheliotropic.

Evidence for strain differentiation
Bonamia exitiosa was originally reported from New Zealand (Dinamani et al. 1983, Hine et al. 2001), but has since been found in Australia (Corbeil et al. 2006a). In the last decade additional unidentified *Bonamia* spp. have been described in other host species and from other locations, Chile (Campalans et al. 2000), Argentina (Kroeck and Montes 2005), the east coast of the USA (Burreson et al. 2004), and Spain (Abollo et al. 2008). Most of the *B.*

exitiosa-like organisms outside Australasia seem closely related to *B. exitiosa*. However, the exact relationship between the "strains" within this *B. exitiosa*-like group is unclear, and species needs to be better defined at the molecular level.

Summary of methods findings:

Four diagnostic techniques have been assessed; heart imprints, histology, PCR (using haplosporidian primers; Carnegie et al. 2000, Cochennec et al. 2000), and ISH (Diggles et al. 2003). ISH was found to be most sensitive, followed by PCR, heart imprints and histology. ISH and PCR (pooled results) detected 96.4% infection, but heart imprints and histology (pooled results) detected 60.7% infection, in the same sample of oysters (Diggles et al. 2003). Histology varies with host species, *B. exitiosa* causing widespread clearly visible infection in *O. chilensis* in New Zealand, but focal necrotic lesions containing cryptic infections in *O. angasi* in Australia. A TaqMan real-time PCR has been developed using Australian *B. exitiosa*, that is *Bonamia* genus-specific (Corbeil et al. 2006b). PCR, heart imprints, and histology can be used for targeted surveillance and presumptive diagnosis, and sequencing is the recommended method for confirmatory diagnosis (OIE 2009).

Gaps in knowledge

As described above for *B. ostreae*, it is not possible to distinguish *B. ostreae* from *B. exitiosa* by histopathology or tissue imprints. Also the described PCR and real time PCR methods are not species specific. There is no information on what environmental conditions favour the parasite.

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Infection with *Bonamia exitiosa*: occurrence and distribution

Review author(s) & Date:

Marc Engelsma
CVI of Wageningen UR, Lelystad, Netherlands

Mike Hine
Cefas, Weymouth, UK

October 2009

Selection of studies:

Sources accessed included, PubMed, Ovid-CAB Abstracts, Ovid-Biological Abstracts, Ovid-Current Contents, Promed, OIE website and WAHID interface and the extensive collections of publications held by the authors. Searches were carried out from May to September 2009.
Search term used was "*Bonamia*" excluding "*Convolvulaceae*" (to exclude the genus *Bonamia* in plant family *Convolvulaceae*).

Data and analyses:

For *Bonamia* 213 documents were retrieved in the Ovid searches. In total 64 documents were used for the review of the *Bonamia* species of which 20 for *Bonamia exitiosa*. The used references are listed below. The extracted data is tabulated in the ***Bonamia exitiosa* Data Entrysheet**. Many other documents give details of parasite ultrastructure, host-parasite inter-relationships, and the effects of stressors. They were not included as they did not give any additional information relevant to geographical occurrence or host species. *Mikrocytos roughleyi* was transferred to the genus *Bonamia* on the basis of a partial SSU rDNA sequence with a very high similarity to *Bonamia exitiosa* (Cochennec-Laureau et al. 2003). However, the sequence was presumably a single positive sample and not definitively linked to the electron micrographs. *Bonamia roughleyi* was therefore not considered in this review.

The quality ratings applied to the evidence presented for occurrence were:

Definite

- In a known susceptible host species within the known geographical range of *Bonamia exitiosa* a definite case of infection with *B. exitiosa* is a positive result by one of the following methods: histopathology, tissue imprints, combined with a positive result by electron microscopy, *in situ* hybridization, PCR, RFLP or sequencing.
- In other host species or outside the known range of *B. exitiosa* a definite case of infection with *B. exitiosa* is a positive result by histopathology or tissue imprints combined with a technique for species identification as electron microscopy, RFLP or sequencing.

Probable

- In a known susceptible host species within the known geographical range of *Bonamia exitiosa* a probable case of infection with *B. exitiosa* is a positive result by one of the following methods: histopathology, tissue imprints, *in situ* hybridization, electron microscopy or PCR.
- In other host species or outside the known range of *B. exitiosa* a probable case of infection with *B. exitiosa* is a positive result by one of the following methods: histopathology, tissue imprints, combined with positive result by PCR or *in situ* hybridization.

Possible

- In other host species or outside the known range of *B. exitiosa* a possible case of infection with *B. exitiosa* is a positive result by one of the following methods: histopathology, tissue imprints, PCR. Furthermore, in other host species or outside the known range of *B. exitiosa* a case can be defined as possible if there is not enough information to type the species to *B. exitiosa*.

Doubtful

- A case is rejected if none of the above described cases is applicable and/or the publication lacks enough details to verify the results of the study.

Summary of disease findings:

The ***Bonamia exitiosa* Data Entrysheet** lists 2 countries in Australasia and 2 hosts *Ostrea chilensis* and *Ostrea angasi* for *Bonamia exitiosa* (*sensu stricto*) (Table 1). In addition 2 countries in South America, 1 in North America and 2 in Europe were listed for *Bonamia exitiosa*-like organisms (*Bonamia exitiosa sensu lato*) with 3 additional host species (*Ostrea edulis*, *Ostrea peulchana*, *Crassostrea ariakensis* and *Ostreola equestris*). The exact relationship between *B. exitiosa sensu stricto and B. exitiosa sensu lato* is currently unclear. It needs to be further investigated whether these geographical isolates belong to the same species, different strains of the same species or if one or more different species can be distinghuised.

Mortalities and importance:

Mortalities among were first observed in 1985, and between then and 1992, the oyster stocks in large beds in Foveaux Strait, south of the South Island, were reduced to 9% of their previous level (Cranfield et al. 2004). Epizootic mortalities have occurred among *O. angasi* in Tasmania, Australia, and in both New Zealand and Australia, attempts at farming oysters in infected areas have resulted in < 100% mortalities.

Possible sources of bias:

As discussed above, the indistinctness of the different geographical isolates belonging to this species hampers the assessment of the distribution of *B. exitiosa*.

Other comments:

None

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Table 1: Countries, locations and overview host species

Country	Locations	Host species
Argentina	San Matías Gulf[$]	*Ostrea peulchana*
Australia	New South Wales; Tasmania[$]	*Ostrea angasi*
Chile	Chiloe Island[$]	*Ostrea chilensis**
New Zealand	Foveaux Strait	*Ostrea chilensis** (=*Tiostrea chilensis*)
Spain	Galicia	*Ostrea edulis*
U.S.A.	North Carolina[$]; Maryland[$]; Virginia[$]	*Crassostrea ariakensis*
		Ostreola equestris

*natural host species
[$]species identity uncertain

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Infection with *Bonamia ostreae*: disease detection, pathogen identification and typing

Review author(s) & Date:

Marc Engelsma
CVI of Wageningen UR, Lelystad, Netherlands

Mike Hine
Cefas, Weymouth, UK

October 2009

Causative agent description:

Characteristics of causative agent

Bonamia ostreae is an intracellular protozoan parasite of the phylum Haplosporidia. This parasite was first described in the European flat oyster (*Ostrea edulis*) by Pichot et al. (1980). It infects haemocytes of the host with the infection occurring disseminated throughout the host. The proliferation of the parasite within the haemocytes will be ultimately lethal for the oyster.

A few studies describe ulcerations and of gill and mantel tissue correlated with infected oysters. However, no gross signs are visible on most of the infected oysters.

Evidence for strain differentiation

Up till now there are no studies indicating differences between *Bonamia* isolates or demonstrating different levels of pathogenicity for different *Bonamia* isolates.

Summary of methods findings:

A range of diagnostic methods has been described for the detection of *B. ostreae*. A number of studies have been directed to the comparison of the different method for detection of *B. ostreae* (e.g. da Silva Villalba 2004, Balseiro et al. 2006, Lynch et al. 2008). For the light microscopy techniques histological sections and heart smears, most often the latter seems to be more sensitive. Though the training background of the personnel could favour for one or the other technique. PCR techniques (Carnegie et al. 2000; Cochennec et al. 2000) alone or in combination with heart smears increases the sensitivity of the detection. Furthermore, a genus specific Taqman real time PCR assay has been developed by Marty et al. (2006). Histology is the golden standard and is the recommended surveillance method by the OIE in regions only infected by *B. ostreae* (OIE 2009). The recommended methods for presumptive diagnosis of infection with *B. ostreae* are, for reasons of availability, utility and diagnostic specificity and sensitivity, tissue imprints and PCR. For confirmatory diagnosis, transmission electron microscopy and sequencing are recommended.

Gaps in knowledge:

Most of the methods described are con-specific for *Bonamia*. It is not possible to distinguish *B. ostreae* from *B. exitiosa* by histopathology or tissue imprints. Furthermore, also the described ISH, PCR and real time PCR methods are not species specific with the exception of the recently described PCR targeting the actin gene (Lopez-Florez et al. 2007). This assay seems to specifically only amplify the actin genes of *B. ostreae*, although further validation of the assay is necessary.

The OIE recommended PCR method of Cochennec et al. (2000) can under certain circumstances lead to false positive results (Engelsma: *unpub. obs.*). The expected product

size is 300 bp while an a-specific fragment of 295 bp can be generated, presumably from host origin. Sequencing of the amplicon to confirm the positive results is obligatory.

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Infection with *Bonamia ostreae*: occurrence and distribution

Review author(s) & Date:

Marc Engelsma
CVI of Wageningen UR, Lelystad, Netherlands

Mike Hine
Cefas, Weymouth, UK

October 2009

Selection of studies:

Sources accessed included, Ovid-CAB Abstracts, Ovid-Biological Abstracts, Ovid-Current Contents, Promed, OIE website and WAHID interface and the extensive collections of publications held by the authors. Searches were carried out from May to September 2009.
Search term used was "*Bonamia*" excluding "*Convolvulaceae*" (to exclude the genus *Bonamia* in plant family *Convolvulaceae*).

Data and analyses:

For *Bonamia* 213 documents were retrieved in the Ovid searches. In total 64 documents were used for the review of the *Bonamia* species of which 44 for *Bonamia ostreae*. The used references are listed below. The extracted data is tabulated in the ***Bonamia ostreae*** **Data Entry sheet**. A large number of epidemiological studies were available from a select number of locations only key papers from these sites were included. Furthermore, of the range of studies focusing on particular aspects of the infection (selection of tolerant oysters, immune responses of the host, etc) only those studies adding data on geographical distribution and/or host species were included.

The quality ratings applied to the evidence presented for occurrence were:

Definite

- In a known susceptible host species within the known geographical range of *Bonamia ostreae* a definite case of infection with *B. ostreae* is a positive result by one of the following methods: histopathology, tissue imprints, combined with a positive result by electron microscopy, *in situ* hybridization, PCR, RFLP or sequencing.
- In other host species or outside the known range of *B. ostreae* a definite case of infection with *B. ostreae* is a positive result by histopathology or tissue imprints combined with a technique for species identification as electron microscopy, RFLP or sequencing.

Probable

- In a known susceptible host species within the known geographical range of *Bonamia ostreae* a probable case of infection with *B. ostreae* is a positive result by one of the following methods: histopathology, tissue imprints, *in situ* hybridization, electron microscopy or PCR.
- In other host species or outside the known range of *B. ostreae* a probable case of infection with *B. ostreae* is a positive result by one of the following methods: histopathology, tissue imprints, combined with positive result by PCR or *in situ* hybridization.

Possible

- In other host species or outside the known range of *B. ostreae* a possible case of infection with *B. ostreae* is a positive result by one of the following methods: histopathology, tissue imprints, PCR. Furthermore, in other host species or outside the known range of *B. ostreae* a case can be defined as possible if there is not enough information to type the species to *B. ostreae*.

Doubtful

- A case is rejected if non- of the above described cases is applicable and/or the publication lacks enough details to verify the results of the study.

Summary of disease findings:

The first mortalities of *Ostrea edulis* caused by *Bonamia ostreae* have been reported in France. The parasite has been detected now at most of the major flat oyster culture areas on the Northern hemisphere. The ***Bonamia ostreae* Data Entry sheet** lists 7 countries in Europe, 2 in North America and one in Africa for *B. ostrea* (Table 1). The natural host for *B. ostreae* is *O. edulis* but 3 additional *Ostrea* species were shown to be susceptible for *B. ostreae* when transferred to *B. ostreae* endemic areas. Furthermore, a *Bonamia*-like parasite was observed in imported *Crassostrea rivularis* (= *C. ariakensis*) at a quarantine facility with inlet water from *a B. ostreae* endemic area (Cochennec et al., 1998). The *Bonamia* species identity was later confirmed to be *B. ostreae* (Arzul pers. obs., EFSA susceptible species report). Several documents suggest *Ostrea denselamellosa* to be a susceptible species for infection with *B. ostreae*, however no studies have actually demonstrated susceptibility of this species to *B. ostreae*. Although several cohabitation studies were carried out with *O. conchaphilia* thus far the species has not been infected with *B. ostreae*.

Mortalities and importance:

Bonamia ostreae in conjunction with *Marteilia refringens* has caused a major decline in the cultivation of flat oysters in Western Europe. Most of the cultivation of flat oysters has now been replaced by cultivation of the Pacific oyster *Crassostrea gigas*.

Possible sources of bias:

A number of research institutes have carried out field studies on the epidemiology at a relative limited number of sites, this causes an overrepresentation of these sites in the *Bonamia* entrysheet.

Other comments:

In the past a number of ostreid species originating from different geographical locations have been tested for their suitability to be cultured at French oyster sites (Pascaul et al.,1991; Bougrier et al., 1986; Grizel et al., 1983). Most of the tested species (*Ostrea puelchana*, *Ostrea angasi* and *Ostrea chilensis*) were found to be susceptible to infections with *B. ostreae* and/or *M. refringens*. As the aim of these studies was foremost to observe the growth characteristics of the oyster species, *Bonamia*-species identification has been limited. As currently *B. exitiosa* or *B. exitiosa*-like parasites have been found at the locations of origin, infection with a *Bonamia*-species from the location of origin cannot be excluded, especially in cases where the oysters were relayed directly (*O. chilensis*).

Table 1: Countries, locations and overview host species

Country	Locations	Host species
Belgium	West-Vlaanderen	*Ostrea edulis**
Canada	British Columbia	*Ostrea edulis**
France	Aquitaine; Basse-Normandie; Bretagne; Charente-Maritime; Pays de la Loire	*Ostrea edulis** *Ostrea angasi* *Ostrea chilensis* *Ostrea peulchana* *Crassostrea ariakansis* (= *Crassostrea rivularis*)
Ireland	Border; South-West; West	*Ostrea edulis**
Morocco	Laayoune province	*Ostrea edulis**
Netherlands	Zeeland	*Ostrea edulis**
Norway	Aust-Agder	*Ostrea edulis**
Spain	Galicia	*Ostrea edulis**
U.S.A.	California; Maine; Washington State	*Ostrea edulis**
United Kingdom	England: Cornwall and Isles of Scilly; Devon; Dorset and Somerset; Essex CC; Hampshire CC; Isle of Wight; Kent CC; West Sussex Wales: South West Wales Scotland: Highlands and Islands	*Ostrea edulis** *Ostrea chilensis* (=*Tiostrea lutaria*)

*natural host species

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Infection with *Marteilia refringens*: disease detection, pathogen identification and typing

Review author(s) & Date:

Marc Engelsma
CVI of Wageningen UR, Lelystad, Netherlands

Mike Hine
Cefas, Weymouth, UK

October 2009

Causative agent description:

Characteristics of causative agent

Marteilia refringens, causative agent of Aber disease, is a paramyxean that infect the digestive epithelium of primarily the European flat oyster (*Ostrea edulis*) and mussels (*Mytilus edulis*, *Mytilus galloprovincialis*) but has also been reported to infect other flat oyster species reared in *M. refringens* endemic areas (*O. chilensis,* Grizel et al. 1983; *O. puelchana*, Pascual et al. 1991; *O. angasi*, Bougrier et al. 1986) and range of other host species in the field*, Solen marginata* (Lopez-Flores et al. 2008a), and *Chamelea gallina* (Lopez-Flores et al. 2008b). Stages of *Martielia-refringens*-like organisms have been observed in *Crassostreae virginica* (Renault et al. 1995) and *Crassostrea gigas* (Montes et al. 1998, Cahour 1979), although the parasite does not seem to develop to mature stages in these hosts. Furthermore, *Marteilia refringens*-like organisms have also been reported from *Cerastoderma edule, Ruditapes decussates, Ruditapes philippinarum, Tapes rhomboids, T. pullastra,* and *Ensis minor* (see Berthe et al. 2004). *M. refringens* also infects a copepod, *Paracartia grani,* which may act as an intermediate host (Audemard et al. 2004). Though the cycle has not been completed yet by showing infection of susceptible hosts from infected copepods. *M. refringens* PCR product has also been found in other copepods (Carrasco et al. 2007), but the parasite has not been visualised in those hosts. Although named for its refringent granules, they are not unique to this species.

Evidence for strain differentiation

It was originally thought that *O. edulis* was infected by *M. refringens,* and *M. galloprovincialis* was infected by a very similar species, *Marteilia maurini* (see Comps et al. 1982). However, a study by Villalba et al. (1993) proposed that they were one species, *M. refringens*. An ultrastructural study by Longshaw et al. (2001) confirmed this and found only minor differences between them, which could have been host mediated. A molecular study be Le Roux et al. (2001), showed that, while SSU rRNA gene sequencing did not show a difference between them, polymorphisms in the ITS region of the rRNA gene showed two different sequences, and it was proposed that two types of *M. refringens* could be recognized, type M and type O. The study of López-Flores et al. (2004) suggests, the form most commonly found in oysters, and the other most commonly found in mussels, to be separate strains of one species, *M. refringens*.

Summary of methods findings:

M. refringens is a relatively easy parasite to find by histology (OIE gold standard) and tissue imprints in moderate to heavy infections. However, specific PCR primers that target the ITS1 region, of *M. refringens* is the method of choice in light infections (Le Roux et al. 2001), or under quarantine before the release of introduced stocks. PCR can be used for targeted surveillance, presumptive and confirmatory diagnosis (OIE 2009). Further confirmatory

diagnosis can be made by sequencing, ISH (Le Roux et al. 1999) or transmission electron microscopy.

Gaps in knowledge

The putative life cycle of *M. refringens* involving only mollusc and copepod hosts still has to be validated: *t*he role of copepods in the life cycles of *M. refringens* and other *Marteilia* spp. needs to be clarified, and *Marteilia* needs to be visualised in copepods or other planktonic organisms. Also, *Marteilia* infections in hosts other than oysters and mussels need to be further studied to determine whether they are *M. refringens* or related *Marteilia* spp.

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Infection with *Marteilia refringens*: occurrence and distribution

Review author(s) & Date

Marc Engelsma
CVI of Wageningen UR, Lelystad, Netherlands

Mike Hine
Cefas, Weymouth, UK

October 2009

Selection of studies

Sources accessed included, PubMed, Ovid-CAB Abstracts, Ovid-Biological Abstracts, Ovid-Current Contents, Promed, OIE website and WAHID interface and the collections of publications held by the authors. Searches were carried out from May to September 2009. Search term used was "*Marteilia refringens*".

Data and analyses

For *Marteilia refringens* 85 documents were retrieved in the Ovid searches. In total 39 documents were used for the review, mainly those with information on geographical distribution, host, and impact (prevalences, mortalities). The used references are listed below. The extracted data is tabulated in the ***Marteilia refringens*** **Data Entrysheet**. Papers repetitively including data on occurrence around France, particularly Brittany, were not included. Furthermore, uncharacterized *Marteilia* species have been observed in other host species outside the known range of *M. refringens*, among other publications, in *Ruditapes philippinarum* (Itoh et al. 2005), these were therefore not included in the review.

The quality ratings applied to the evidence presented for occurrence were:

- **Definite**: full details of identification methods were given or confirmation came in a subsequent paper or confirmation was done in a second laboratory; validated methods used.
- **Probable**: Most details of identification were given; validated methods used.
- **Possible**: Some details of identification given; no confirmation reported.
- **Doubtful**: Scant or no details given and or non validated methods used; no confirmation reported.

Summary of disease finding

The ***Marteilia refringens*** **Data Entrysheet** lists 7 countries, 6 in Europe and 1 in Africa and 15 host species from different bivalve genera (Table 1). *Marteilia refringens* causes serious disease in farmed oysters (*Ostrea edulis*) and to a lesser extent in farmed mussels (*Mytilus galloprovincialis*), but also occurs, often at sub-clinical levels, in wild stocks of these species. Possibly other species of molluscs around farm sites may act as reservoirs of infection, and farming may increase levels of infection in nearby reservoir hosts.

Mortalities and importance

Marteilia refringens has had a significant impact on the European *O. edulis* industry. Studies on sites heavily infected by *M. refringens* overall show that infection levels and mortalities

increase from spring to summer as temperatures rise, resulting in mortalities of < 100% at farm sites, although epizootic mortalities under culture may vary from 80-100%. However, some studies have observed peak mortalities in early summer, or rises in infection and mortality during late autumn. Because of these high mortalities under culture, *M. refringens* must be regarded as an important pathogen that may make culture impossible at some sites. In addition, the crowded conditions of culture probably build up levels of infection in the environment surrounding the farm, making culture progressively more difficult.

Possible sources of bias

The OIE diagnostic manual (OIE 2009) reports the occurrence of *M. refringens* in Albania and United Kingdom, however no publications were available for review nor were there any OIE notifications on the occurrence in these two countries. Similarly, *Ostrea denselamellosa* has been deployed in the past in *M. refringens* endemic areas in France, however the report on this was not available for review.

The list of countries and locations does not necessarily represent the current distribution of the parasite. *Marteilia refringens* was introduced in the Netherlands after importation of infected oysters from France in 1974. However, *M. refringens* has not been observed in the Netherlands after 1978 (Engelsma *pers. obs.*).

Other comments

None

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Table 1: Countries, locations and overview host species

Country	**Locations**	**Host species**
Croatia	-	*Mytilus galloprovincialis*
France	Bretagne; Charente-Maritime; Aquitaine	*Ostrea edulis* *Ostrea angasi* *Ostrea chilensis* *Ostrea peulchana* *Crassostrea gigas* *Crassostrea virginica* *Mytilus edulis* *Mytilus galloprovincialis* *Cerastoderma edule* (=*Cardium edule*) *Tapes pullastra* *Tapes rhomboids* *Paracartia grani*
Greece	Gulf of Thessalonika	*Ostrea edulis* *Mytilus galloprovincialis*
Italy	Venezia; Taranto; Tyrrhenian sea	*Mytilus galloprovincialis* *Ensis minor*
Morocco	Nador province	*Ostrea edulis*
Spain	Galicia; Mallorca; Andalucía; Cataluña	*Ostrea edulis* *Mytilus galloprovincialis*

		Crassostrea gigas *Solen marginatus* *Chamelea gallina*
The Netherlands	Zeeland	*Ostrea edulis*

*natural host species

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Infection with *Mikrocytos mackini*: disease detection, pathogen identification and typing

Review author(s) & Date

Marc Engelsma
CVI of Wageningen UR, Lelystad, Netherlands

Mike Hine
Cefas, Weymouth, UK

October 2009

Causative agent description

Characteristics of causative agent
Mikrocytos mackini, the causative agent of Denman Island disease, is a protist of unknown affinities that causes mantle recession, pale digestive gland, shell pustules, abcesses and ulcers (Farley et al. 1988). It is not related to any other known protistan taxa (Carnegie et al. 2003). The parasite causes a focal infection of the vesicular connective tissue, haemocytes, adductor and heart myocytes (Hine et al. 2001) of *Crassostrea gigas*. Although *C. gigas* is the species most commonly infected in the wild, it can also infect *Ostrea edulis, Ostrea conchaphila,* and *Crassostrea virginica* (Bower et al. 1994, 1997). Extended periods with water temperatures below 10ºC are necessary for the development of the disease.

Evidence for strain differentiation
There is no evidence of the existence of strains, but this has not been investigated.

Summary of methods findings

Gross signs of greenish to yellow pustular lesions can be used as a presumptive indicator of infection, if seen on *C. gigas* in a known infected area. However, outside known infected areas and in other hosts these signs are not reliable. Light infections can be very difficult to diagnose, although the parasite may be concentrated by purification (Joly et al. 2001). In low level infections histology can be more reliable than tissue imprints, but otherwise tissue imprints are more sensitive than histology (Hervio et al. 1996). An *M. mackini* specific PCR and FISH have been developed that detect 15 times more *M. mackini* than either tissue imprints or histology (Carnegie et al. 2003). An ISH Digoxigenin-labeled probe has been used to validate the specificity and sensitivity against histology, showing that only 70% of positive oysters diagnosed with ISH were positive by histology (Meyer et al. 2005). Currently *M. mackini* is not regarded in the OIE manual of diagnostic tests (OIE 2009) but in the previous version of the manual (OIE 2006) the OIE recommended histopathology for surveillance and presumptive diagnosis. For confirmatory diagnosis sequencing and transmission electron microscopy were recommended.

Gaps in knowledge

Relatively little is known about *M. mackini* because it has, until recently, been very difficult to detect, and no other closely related organisms are known from which insight may be gained. The developmental cycle has only been put together by studying electron micrographs, and has not been validated. Although a long period of cold seems to be required to bring on the disease, nothing is known about the survival in the wild outside the host.

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Infection with *Mikrocytos mackini*: occurrence and distribution

Review author(s) & Date

Marc Engelsma
CVI of Wageningen UR, Lelystad, Netherlands

Mike Hine
Cefas, Weymouth, UK

October 2009

Selection of studies

Sources accessed included, PubMed, Ovid-CAB Abstracts, Ovid-Biological Abstracts, Ovid-Current Contents, ProMed, OIE website and WAHID interface and the collections of publications held by the authors. Searches were carried out from May to September 2009. Search terms used were "*Mikrocytos mackini*" and "*Microcytos mackini*".

Data and analyses

For *Mikrocytos mackini* 24 documents were retrieved in the Ovid searches. In total 9 documents were used for the review. The used references are listed below. The extracted data is tabulated in the ***Mikrocytos mackini* Data Entry sheet**. The documents analysed reflect the paucity of literature on this parasite, relevant to this study. The several one page conference abstracts published in Journal of Shellfish Research were not included.

The quality ratings applied to the evidence presented for occurrence were:

- **Definite**: full details of identification methods were given or confirmation came in a subsequent paper or confirmation was done in a second laboratory; validated methods used.
- **Probable**: Most details of identification were given; validated methods used.
- **Possible**: Some details of identification given; no confirmation reported.
- **Doubtful**: Scant or no details given and or non validated methods used; no confirmation reported.

Summary of disease findings

Mikrocytos mackini causes localised mortalities. The ***Mikrocytos mackini* Data Entry sheet** lists 2 countries in North America and 4 different host species. It occurs around islands in the Strait of Georgia between Vancouver Island, mainland Canada and Washington State, U.S.A (Table 1). As well as being geographically isolated, it only commonly occurs in *Crassostrea gigas*. However, it is also able to infect *Ostrea edulis, Ostrea conchaphila* and *Crassostrea virginica* under experimental conditions. This geographical isolation and host restriction may be because it requires a long period of cold before the disease becomes manifest. A *Mikrocytos*-like organism has recently been detected in *O. edulis* originating from Nova Scotia, on the Atlantic seaboard of Canada (Gagné et al. 2008), which is also a cold region.

Mortalities and importance

Disease outbreaks seem to be sporadic and localised, with mortality patterns changing year by year. During the outbreak at Henry Bay, Denman Island, in 1960, monthly mortalities were

~10% (April), 10% (mid-May), ~40% (late May), and in early August 17% (at the 4 foot tide level), 30% (at the 2.5 foot tide level and 53% (at 1 foot tide level), with a mean of 34% (Quayle 1961). Later the mortalities were reported as 17-53%, and ~25% overall (Quayle 1982). Mortality rates varied greatly from bay to bay. Looking at these and subsequent mortalities, it appears that Denman Island Disease is a localised, geographically restricted disease that fluctuates year by year, producing moderate scale mortalities.

Possible sources of bias

The relationship of *M. mackini* with the *Mikrocytos*-like organism detected in imported *O. edulis* from Nova Scotia (Gagné et al. 2008) is not clear. The latter could concern another *Mikrocytos* species.

Other comments

None

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Table 1: Countries and locations

Country	Locations	Host species
Canada	British Columbia	*Crassostrea gigas**
		Crassostrea virginica
		Ostrea conchaphila
		Ostrea edulis
USA	Washington State	*Crassostrea gigas**

*natural host species

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Infection with *Perkinsus marinus*: disease detection, pathogen identification and typing

Review author(s) & Date

Marc Engelsma
CVI of Wageningen UR, Lelystad, Netherlands

Mike Hine
Cefas, Weymouth, UK

October 2009

Causative agent description

Characteristics of causative agent

Perkinsus marinus is a dinoflagellate-like organism (Reece et al. 1997, Bushek et al. 2002) and causative agent of dermo disease in the American oyster *Crassostrea virginica*. Under experimental conditions it has also been shown to infect the oyster species *Crassostrea gigas, Crassostrea ariakensis* (Calvo et al. 1999, 2001) and the clams *Mya arenaria* and *Macoma balthica* (Dungan et al. 2007). Furthermore, it has also been reported in *Crassostrea rhizophorae* (Littlewood 2000) and *Crassostrea corteziensis* (Cáceres-Martínez et al. 2008). *Perkinsus marinus* can be isolated from the environment as well as from infected oysters, suggesting that it may act as a facultative parasite. The prevalence and intensity of the infection is dependent on the salinity, higher salinity increases the infection and intensity. While the annual cycle of P. marinus follows the water temperature (Burreson Calvo, 1996).

Evidence for strain differentiation

There is good evidence for the existence of 'races' of *P. marinus* occurring over the long geographical distances of the parasite's range (Bushek & Allen 1996). Molecular genetics shows that distinct strains occur in 3 regions, the northeast Atlantic (Maine to Maryland), the southeast Atlantic (Virginia to east coast Florida), and the Gulf of Mexico (west coast Florida to Texas). Twelve different composite genotypes are known, with 88% of isolates possessing one of 3 dominant genotypes. One of the major composite genotypes only occurs in Gulf isolates, while the most frequent genetic strain in the northeast Atlantic does not occur in Gulf coast oysters (Reece et al. 2001). Another study found 2 strains of *P. marinus* which in the NTS region differed at 6 nucleotide positions. Type I was found in Maryland, type II in Florida, and both types in Louisiana (Robledo et al. 1999). *Perkinsus* strains may adapt to different environmental conditions, such as the adaptation of Long Island strains to high salinity, and Chesapeake Bay isolates to low and variable salinities (Yee et al. 2005). *Perkinsus* strains may also differ in surface lectins (Gauthier et al. 2004).

Summary of methods findings

Several diagnostic techniques have been developed, but their utility differs depending on whether diagnosis is in the field or in the laboratory, whether it is presumptive or confirmatory, and whether cost is a factor. Ray's fluid thioglycollate medium (RFTM) cultures are most widely used. The RFTM body burden assay (Choi et al. 1989, Fisher Oliver 1996) is the golden standard technique for the OIE (2009) while the RFTM tissue assay (Ray 1966) is the recommended technique for targeted surveillance. However, the assays are genus specific and not specific for *P. marinus*, only. PCR with *Perkinsus* genus specific primers and *P. marinus* specific primers (Audemard et al. 2004) is recommended for presumptive diagnosis. Subsequent use of *in situ* hybridisation may be regarded as confirmatory (OIE 2009).

Gaps in knowledge

Current diagnostic methods of utility in the field (RFTM cultures) are non-specific for *P. marinus*, and a more specific test is needed for confirmation. Other *Perkinsus* species need to be better defined to allow for sensitive and specific molecular tests to discriminate *P. marinus*. While the parasite has been studied extensively *in vivo* and *in vitro,* more information is needed on *P. marinus* as an environmental contaminant.

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Infection with *Perkinsus marinus*: occurrence and distribution

Review author(s) & Date

Marc Engelsma
CVI of Wageningen UR, Lelystad, Netherlands

Mike Hine
Cefas, Weymouth, UK

October 2009

Selection of studies

Sources accessed included, PubMed, Ovid-CAB Abstracts, Ovid-Biological Abstracts, Ovid-Current Contents, Promed, OIE website and WAHID interface and the collections of publications held by the authors. Searches were carried out from May to September 2009.
Search terms used were "*Perkinsus marinus*" and its symonym "*Dermocystidium marinum*".

Data and analyses

For *Perkinsus marinus* 557 documents were retrieved in the Ovid searches. In total 22 documents were used for the review. The used references are listed below. The extracted data is tabulated in ***Perkinsus marinus* Data Entry sheet**. Although there many more documents giving details of geographical occurrence, they largely overlap with those that were chosen. A number of papers on strains were not analysed as they largely dealt with techniques, rather than field results.

The quality ratings applied to the evidence presented for occurrence were:

- **Definite**: full details of identification methods were given or confirmation came in a subsequent paper or confirmation was done in a second laboratory; validated methods used.
- **Probable**: Most details of identification were given; validated methods used.
- **Possible**: Some details of identification given; no confirmation reported.
- **Doubtful**: Scant or no details given and or non validated methods used; no confirmation reported.

Summary of disease findings

The ***Perkinsus marinus* Data Entry sheet** lists 7 countries in the America's and 7 host species (Table 1). *Perkinsus marinus* causes serious disease in *Crassostrea virginica,* at high temperatures and high salinities. Mortalities during epizootics are often > 95% among *C. virginica,* but the other susceptible hosts do not suffer the same mortalities. Less susceptible hosts may act as reservoir of infection, and *P. marinus* can be isolated from water samples (Audemard et al. 2004). There is varying susceptibility among different *C. virginica* populations, and this may be related to host genetics, as well as the existence of several strains of the parasite. The parasite occurs along the eastern coast of the United States and has been reported from every coastal state from Massachusetts (Cape Cod) to Texas (Corpus Christi Bay), and around the Gulf of Mexico. The parasite appears to be ubiquitous over that range. At geographical locations with *P. marinus* the prevalence of the infection in *C. virginica* is often high to very high. Subsequently, the intensity of the infection is often an

important factor in estimating the parasite burden on the population. *P. marinus* also occurs around the Gulf of Mexico to the Yucatan Peninsula, and has been reported from Nayarit on the Pacific coast of Mexico. The reports from Brazil, Cuba, Puerto Rico and Venezuela lack details to be assessed.

Mortalities and importance

Perkinsus marinus and *Haplosporidium nelsoni* have reduced oyster production in Chesapeake Bay to the extent that current production is < 20% of what it was in the early 1950s. To try to overcome this, consideration and a lot of funding have been given to evaluate other oyster species as candidates for aquaculture. These include *Crassostrea gigas* and *Crassostrea ariakensis,* but no decisions have been made for fear that they may introduce exotic diseases, despite rigorous quarantine, or may cause environmental damage by uncontrolled spread, as has been seen with *C. gigas* in other countries. At present it appears that *P. marinus* will continue to impact on *C. virginica* farming unless resistant oyster stocks can be developed, and such stocks farmed in environments unfavourable to the pathogen.

Possible sources of bias

None are apparent. *P. marinus* has been intensively studied, and reports and research have been consistent in their findings.

Other comments

None

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Table 1: Countries, locations and overview host species

Country	Locations	Host species
Brazil	-	*Crassostrea virginica**
Cuba	-	*Crassostrea virginica**
Jamaica	-	*Crassostrea rhizophorae*
Mexico	Campeche; Nayarit; Tabasco; Veracruz	*Crassostrea virginica**
		Crassostrea corteziensis
Puerto Rico	-	*Crassostrea virginica**
USA	Alabama; Connecticut; Florida; Georgia; Hawaii; Louisiana; Maryland; Massachusetts; Mississippi; New Jersey; New York; Rhode Island; South Carolina; Texas; Virginia	*Crassostrea virginica**
		Crassostrea gigas
		Crassostrea ariakensis
		Mya arenaria
		Macoma balthica
Venezuela	-	*Crassostrea virginica**

*natural host species

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3) Crustacean diseases

Taura syndrome: disease detection, pathogen identification and typing

Review author(s) & Date

Dr Grant Stentiford
Cefas, Weymouth, United Kingdom

October 2009

Causative agent description

Taura Syndrome is considered to be infection with Taura Syndrome Virus (TSV). TSV is listed as an unassigned species in the family *Dicistroviridae*. Virus particles are non-enveloped icosahedrons measuring 30-32nm and replicate within the cytoplasm of host cells. TSV consists of a linear, positive sense ssRNA with a buoyant density of 1.338g/ml. At least three genotypic variants have been identified based upon the sequence of the capsid protein VP1. These genotypic groups are 1) the Americas group; 2) the South-East Asia group; and 3) the Belize group. Monoclonal antibody MAb 1A1 produced to a reference isolate from the Americas has demonstrated at least two distinct variants. Type A are those that react with MAb 1A1. Those that do not react with MAb 1A1 have been further divided into Type B (TSV 98 Sinaloa and Mexico) and Type C (TSV 02 Belize) based on host species and virulence. All TSV isolates from the Americas and most South-East Asian genotypes react with MAb 1A1. None of the Belize genotype group react with MAb 1A1 and neither does a 2005 TSV isolate from Venezuela. In Ecuador Taura Syndrome was initially linked to pesticide contamination of shrimp farms, a contention that was supported by litigation for ~8 years after the disease was shown to be viral. Hence several papers in the literature propose a toxic aetiology for the syndrome.

TS is known as a nursery or post larval (PL) disease occurring 14-40 days after stocking of PL's. *In vivo* titration has not been performed for TSV and as such, infectious dose is not documented. TSV can be transmitted by horizontal transmission or by contaminated water. Vertical transmission is suspected but has not been experimentally confirmed.

In regions where the virus is enzootic in farmed stocks the prevalence can range from 0-100%. TS is widely distributed in the shrimp farming regions of the Americas and South-East Asia. TSV has been shown to remain infectious for up to 48 hours in the faeces passed by wild or captive sea gulls after consuming TSV infected shrimp carcasses. Water insects may act as mechanical vectors for TSV. Frozen TSV-infected commodity products have been shown to have the potential to contaminate wild or farmed stocks.

On-farm epizootics of TS involving *Penaeus vannamei* (principle host species) typically result in cumulative mortalities of between 40 and 100 %. Survivors of TS outbreaks may carry the virus for life. No effective vaccines for TSV are available. TSV-resistant stocks of *L. vannamei* and *L. stylirostris* have been reported. Use of specific pathogen free (SPF) or PCR negative seed stocks and use of biosecure water and culture systems.

A comprehensive assessment of host susceptibility to TS has recently been completed by EFSA (EFSA 2008). This data is summarised in Stentiford et al. (2009). The assessment takes in to account the key susceptibility criteria of pathogen replication, bioassay, characteristic pathology and anatomical location of pathogen and has critically assessed the available literature on host-pathogen interaction with respect to TS. Gulf white shrimp

(*Penaeus setiferus*), Pacific blue shrimp (*P. stylirostris*), and Pacific white shrimp (*P. vannamei*) are currently listed as species susceptible to TS virus in the Directive 2006/88/EC. However, the EFSA assessment identified scientific literature to confirm susceptibility of *P. vannamei, P. duorarum, P. monodon, P. setiferus, P. chinensis, P. stylirostris, P. aztecus,* and *Metapenaeus ensis*. Some information is available to also suggest susceptibility of *P. schmitti* and *P. japonicus* though not all of the EFSA criteria were met in these cases.

Summary of methods findings

Gross signs

Penaeid shrimp. TSV has three distinct phases, which are grossly distinguishable:

Acute - shrimp have a general pale reddish colouration with the tail fan and pleopods appearing hyperpigmented (red) due to the expansion of chromatophores. Typically the cuticle is soft and the gut empty, and infected shrimps may not survive ecdysis. Moribund shrimp accumulate at the pond surfaces and edges. During outbreaks, birds may be seen feeding on moribund shrimp.

Transition (Recovery) – Shrimp exhibit random, multifocal, irregularly shaped melanised cuticular lesions. These melanised spots are haemocytic aggregations indicating sites resolving from TSV infection. These shrimp may or may not have soft cuticle and red-chromatophore expansion, and may feed and behave normally.

Chronic – Following successfully moulting, shrimp move into the chronic phase of TS in which animals show no obvious signs of disease. Chronically infected shrimp may however be less resistant to normal environmental stressors than uninfected shrimp and appear to become persistent carriers.

Crabs, crayfish, freshwater prawns, spiny lobsters and clawed lobsters. Unknown susceptibility and pathological outcome in the majority of species.

Clinical Pathology

Histology. In penaeid shrimp, TSV has been shown to infect the cuticular epithelium, foregut, hindgut, gills, appendages, haematopoietic tissues, lymphoid organ and antennal gland. The hepatopancreas, midgut, midgut caeca, smooth cardiac muscle, striated muscle, ventral nerve cord and ganglia typically show no histological signs of infection. Histology has not been well described for TS infections of other crustaceans.

Acute – Multifocal areas of necrosis in the cuticular epithelium and epithelium of the gills, hindgut and foregut. Affected cells display increased eosinophillia of the cytoplasm and pyknotic or karyorrhectic nuclei. Cytoplasmic remnants of necrotic cells are often present in these acute phase lesions and appear as spherical bodies (1 - 20 µm diameter) that range in staining from eosinophilic to pale basophilic. These structures along with pyknotic and karyorrhectic nuclei give the lesions a “peppered” or “buckshot-riddled” appearance. In TSV infected tissues pyknotic or karyorrhectic nuclei stain positively with Feulgen stain distinguishing them from less basophilic to eosinophillic cytoplasmic inclusions that do not contain DNA. The absence of necrosis of the lymphoid organ in TSV infections distinguishes TS
disease from acute Yellowhead disease.

Transition (Recovery) – Cuticular lesions decline in abundance and severity and are replaced by infiltration and accumulation of haemocytes at the sites of necrosis. Haemocyte aggregates progress to melanised granulomatous lesions giving rise to irregular black spots on the cuticle that characterise the transition phase of the disease. Lesions may show erosion

of the cuticle, surface colonisation and invasion of the affected cuticle and exposed haemocytes with secondary pathogens (e.g. *Vibrio* spp.).

Chronic – Presence of numerous prominent lymphoid organ spheroids (LOS) which may remain associated with the main body of the lymphoid organ or which may detach and become ectopic LOS bodies that lodge in constricted areas of the haemocoel (heart, gills, subcuticular connective tissues etc).

*In situ hybridisation (ISH)***:** *Transition* - Sections of lymphoid organ may appear normal when stained with H&E however when the same tissue is assayed for TSV by ISH, TSV aggregates are shown in peripheral parenchymal cells of the lymphoid organ tubules. *Chronic* – When assayed by ISH, some cells within the LOS give positive reactions for the virus while no other target tissues react. A cDNA probe for TSV provides excellent diagnostic sensitivity via the nonradioactive, DIG-labelled probe used in dot blot and *in situ* hybridisation assays (see most recent OIE Manual of Diagnostic Tests for Aquatic Animals).

RT-PCR: An RT-PCR method that amplifies a 231bp fragment of the TSV genome is reported by Nunan et al. (1998) while a real time PCR assay with increased speed, sensitivity and specificity is reported by Tang et al. (2004). Both techniques are detailed in the most recent OIE Manual of Diagnostic Tests for Aquatic Animals. Confirmation of TSV can be achieved by nucleotide sequencing of the PCR product.

*Sequencing***.** For confirmation of TSV, the amplified product from the RT-PCR assay should undergo nucleotide sequencing. If a positive result is obtained, compare the sequences to available databases using the Basic Local Alignment Search Tool (BLAST) to determine approximate phylogenetic affiliations. If a negative result is obtained the sample should be tested again.

Gaps in knowledge

At least four genotypic groups of TSV have been identified based on the sequence of the VP1 (=CP2) capsid protein – the largest and presumably most dominant protein of the virus. Based upon the VP1 sequences (generally those sequences lodged in GenBank), the genogroups are: 1. the Americas group, 2. the South-East Asian group; 3. the Belize group and 4. The Venezuala group. The groupings affect abilities to detect TSV by recognised molecular and antigenic diagnostic tests (OIE, 2006). Many other isolates are probably in use in various laboratories that relate to outbreaks of TSV globally. In the Americas alone, outbreaks have been reported from Ecuador, Colombia, Peru, Brazil, El Salvador, Guatamala, Honduras, Mexico, Belize, Nicaragua, Panama, Costa Rica and from various US State. To date, little work has been carried out on the differential pathogenicity of these different strains and importantly, we have no information on their potential for infection of susceptible crustacean species residing in European aquatic habitats. Similar to the case for the YHD complex viruses, the TS viruses may eventually be shown to have differential virulence in novel hosts and environments and as such, use of detection methods suitable for all (known) isolates are recommended.

Due to the demonstrable survival of TSV in raw frozen commodity products arising from infected harvests, there is an important gap in knowledge regarding the potential for infectious doses (to different species) within products of different types and furthermore, the potential for exposure to, and consequence for, naïve populations of susceptible species. Furthermore, countries receiving TSV-infected frozen raw commodity products will likely contain infected products in retail outlets and in processing establishments.

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Taura syndrome: occurrence and distribution

Review author(s) & Date

Dr Grant Stentiford
Cefas, Weymouth, United Kingdom

October 2009

Selection of studies

ASFA and Scopus were databases most frequently used. Search terms included: TS, TSV, Taura syndrome, Taura syndrome virus, ICENV, epithelial viral necrosis. Databases were searched during 2009.

Several reports of TS were considered to be in "grey" literature (including reports from the OIE/WAHID database). In the latter cases, the evidence, particularly where confirmation of disease agent occurred at an OIE reference laboratory would be taken as "Definite".

Data and analyses

Approximately 150 documents were appraised, and data from approximately 20 were entered into the data table. The aim was to include studies reporting the isolation/detection of the virus, particularly if they reported a new country or new host species. Papers recording the detection of the virus based on PCR alone were in general not included, and definitely not if sequencing was not done. Some of the studies appraised collections of a number of isolates from different sources and then compared them by molecular biology methods. Most often the only information about an isolate was host species, location and year of isolation although all three attributes may not have been reported. Such studies may not have been included unless they added a new host or country, or added data to another publication already in the data table.

The quality ratings applied to the evidence presented for occurrence were:

- **Definite**: full details of identification methods were given or confirmation came in a subsequent paper or confirmation was done in a second laboratory; validated methods used.
- **Probable**: Most details of identification were given; validated methods used.
- **Possible**: Some details of identification given; no confirmation reported.
- **Doubtful**: Scant or no details given and or non validated methods used; no confirmation reported.

Summary of disease findings

Initially interpreted as a toxicological problem with mortalities due to fungicides used in the treatment of banana trees in the region, a viral aetiology was demonstrated a few years later; the causative agent named Taura Syndrome Virus (TSV). It is suspected by farmers that the disease was already present in this area by the mid-1980s. Originally limited to the Americas, more recently the disease has been reported in Asia following its introduction with infected imported P. vannamei from Central and South America. The agent is a small nonenveloped ssRNA icosahedral virus, 32 nm in diameter, developing in the cytoplasm of tissues of ectodermic and mesodermic origin. It is classified in the Dicistroviridae family. Several studies have not sequenced the amplicon from RT-PCR and as such it is difficult to ascertain the true divergence in global isolates of this virus.

Other comments

Appraisal of the documents often revealed the lack of information about the circumstances of the isolation (e.g. accurate location, level of mortality, genotyping of virus). An outcome of this review could be a standardised system for describing disease outbreaks in the field and also, a standardised assessment of susceptibility in novel hosts by fulfilment of EFSA criteria.

White Spot Disease: disease detection, pathogen identification and typing

Review author(s) & Date:

Dr Grant Stentiford
Cefas, Weymouth, United Kingdom

October 2009

Causative agent description:

White Spot Disease (WSD) is considered to be infection with the virus White Spot Syndrome Virus-1 (WSSV). Virus particles appear rod-shaped to elliptical and measure 80-120 x 250-380 nm. WSSV belongs to the *Whispovirus* genus in the *Nimaviridae* Family. WSSV is a dsDNA virus with a 293kb genome. WSD is the most serious threat facing the shrimp farming industry. The economic impact of the disease in value of lost production and trade has been reported to approach 10 billion USD since 1993.

Vertical (trans-ovum), horizontal (cannibalism, predation etc.) and water-borne transmission routes likely. Transmission can occur from apparently healthy animals in the absence of disease. Dead and moribund animals may be a source of disease transmission. Geographic distribution throughout East, South-East and South Asia, North, South and Central America. WSD free zones are known within these regions. Reported outbreaks in European shrimp production regions. Rotifers, bivalves, polychaete worms and non-decapod crustacean hosts including *Artemia salina* and copepods, non-crustacean aquatic arthropods and insect larvae. All these species can accumulate high concentrations of viable WSSV but there is no evidence of virus replication. Likely that any animal in contact with pond water and/or feeding/in contact with infected hosts may act as a vector for WSSV.

All farmed penaeid shrimp species are highly susceptible to infection often resulting in high mortality. Crabs, crayfish, freshwater prawns, spiny lobsters and clawed lobsters are susceptible to infection but mortality and morbidity is highly variable. Disease outbreaks may be induced by stressors (for example a rapid change in salinity). Water temperature has a profound effect on disease expression with average water temperatures of below ~30ºC being conducive to WSD outbreaks. Lower temperature limits of WSD expression and transfer of hosts from 'carrier' to 'diseased' status have not been well studied, particularly for temperate water species. No effective vaccines for WSSV are available. A comprehensive assessment of host susceptibility to WSD has recently been completed by EFSA (EFSA 2008). This data is summarised in Stentiford et al. (2009).

Summary of methods findings:

Gross Pathology

Penaeid shrimp. Presence of white spots under the cuticle and a high degree of colour variation with a predominance of reddish and pinkish discoloured shrimp, reduction in feed intake, increased lethargy, movement of moribund shrimp to the water surface and pond/tank edges and consequent attraction of shrimp-eating birds. Note: all of these signs are generally considered non-specific and as such cannot be used as definitive for a diagnosis of WSD.

Crabs, crayfish, freshwater prawns, spiny lobsters and clawed lobsters. Gross symptoms of WSD in non-penaeid crustacean hosts are not well documented but are likely to include at least a reduction in feed intake, an onset of lethargy and other behavioural changes. White

spots beneath the cuticle may be unlikely due to the thickness of the carapace of most species.

Clinical Pathology

*Histology***.** H&E staining reveals intranuclear inclusion bodies as prominent eosinophilic to pale basophilic in hypertrophied nuclei. Most commonly seen in the cuticular epithelial cells and connective tissue cells, and, less frequently, the antennal gland epithelium, lymphoid organ sheath cells, haematopoietic cells and fixed phagocytes of the heart. Feulgen staining reveals the intranuclear inclusion bodies to be Feulgen positive. Intranuclear occlusion bodies are absent (Annex 1).

Transmission Electron Microscopy (TEM). WSSV particles can be seen within the intranuclear inclusion bodies of infected cells. Virions are rod-shaped to elliptical, non-occluded and measure between 80 - 120 nm in width and 250 - 380 nm in length (Annex 2).

Polymerase Chain Reaction (PCR). The suggested protocol is that described by Lo *et al.* (1997 & 1996), and is recommended for all situations where WSSV diagnosis is required. A positive result in the first step of this standard protocol implies an advanced WSSV infection; when a positive result is obtained in the second amplification step only, a latent or carrierstate infection is indicated. Commercial PCR diagnostic kits are available and have been very useful in the standardization and harmonisation of the technique. It is recommended that the most recent OIE Diagnostic Manual be consulted for up-to-date developments in molecular diagnostics for WSD.

In situ hybridisation (ISH). WSSV infected nuclei can be intensely marked by a DIG-labelled DNA probe for WSSV with *in situ* hybridisation assays. The suggested protocol is that developed by Nunan & Lightner (1997).

Polymerase Chain Reaction (PCR). As for surveillance. Follow protocol of Lo *et al.* (1996, 1997) or most recent OIE Diagnostic Manual.

Sequencing. For confirmation of suspected WSSV, the DNA fragment amplified from the two-step nested diagnostic PCR should be sequenced. The suggested cloning and sequencing protocols are those described by Claydon *et al.* (2004). It is acceptable to sequence the PCR amplicon directly. If a positive result is obtained, compare the sequences to available databases using the Basic Local Alignment Search Tool (BLAST) to determine approximate phylogenetic affiliations. If a negative result is obtained the sample should be tested again.

Gaps in knowledge

Despite being perhaps the best studied of the crustacean viruses, there are several gaps in knowledge associated with genotypes of WSSV and the presence of other WSSV-like pathogens in crustacean hosts. To date, only one genotype (WSSV-1) has been described, with all isolates being placed into this group. However, studies which have applied the standard diagnostic tests (particularly PCR) to new hosts in farmed and wild locations have often failed to carry out sequencing of the amplicon and have thus assumed that the amplified product is from WSSV (and further, WSSV-1). Older studies in Europe and the USA have also discovered viruses using histology/TEM which appear morphologically indistinct from WSSV but have never been analysed using molecular phylogenetic tools. It could be envisaged that further isolates of WSSV (i.e. WSSV-n) may be discovered by utilising the appropriate diagnostic tools for new descriptions and further that some current detections may in fact be different genotypes to WSSV-1.

Due to the demonstrable survival of WSSV in raw frozen commodity products arising from infected harvests, there is an important gap in knowledge regarding the potential for infectious doses (to different species) within products of different types and furthermore, the potential for exposure to, and consequence for, naïve populations of susceptible species. Furthermore, countries receiving WSSV-infected frozen raw commodity products will likely contain infected products in retail outlets and in processing establishments.

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White Spot Disease: occurrence and distribution

Review author(s) & Date

Dr Grant Stentiford
Cefas, Weymouth, United Kingdom

October 2009

Selection of studies

ASFA and Scopus were databases most frequently used. Search terms included: WSSV, WSD, white spot syndrome virus, white spot disease, PAV, penaeid acute viraemia/viraemia, RV-PJ, rod shaped virus of Penaeus japonicus etc. Databases were searched during 2009.

Several reports of WSD/WSSV were considered to be in "grey" literature (including reports from the OIE/WAHID database). In the latter cases, the evidence, particularly where confirmation of disease agent occurred at an OIE reference laboratory would be taken as "Definite".

Data and analyses:

Over 500 documents were appraised, and data from approximately 70 were entered into the data table. The aim was to include studies reporting the isolation/detection of the virus, particularly if they reported a new country or new host species. Papers recording the detection of the virus based on PCR alone were in general not included, and definitely not if sequencing was not done. Some of the studies appraised collections of a number of isolates from different sources and then compared them by molecular biology methods. Most often the only information about an isolate was host species, location and year of isolation although all three attributes may not have been reported. Such studies may not have been included unless they added a new host or country, or added data to another publication already in the data table.

The quality ratings applied to the evidence presented for occurrence were:

- **Definite**: full details of identification methods were given or confirmation came in a subsequent paper or confirmation was done in a second laboratory; validated methods used.
- **Probable**: Most details of identification were given; validated methods used.
- **Possible**: Some details of identification given; no confirmation reported.
- **Doubtful**: Scant or no details given and or non validated methods used; no confirmation reported.

Summary of disease findings

The disease originally occurred in Japan and Taiwan in *Penaeus japonicus* The virus received different names during the first years after it appeared, these including hypodermal and haematopoietic necrosis baculovirus (HHNBV), rod-shaped nuclear virus of P. japonicus (RV-PJ), systemic ectodermal and mesodermal baculovirus (SEMBV), white spot baculovirus (WSBV), and Chinese baculovirus (CBV). All of these isolates are now recognised as one virus:WSSV, with the three isolates fully sequenced revealing only minor differences, related to a single deletion (GenBank Accession No AF332093, GenBank Accession No AF369029 and GenBank Accession No AF440570). This deletion however may have relevance when

assessing the virulence of these isolates. Many studies have not sequenced the amplicon from PCR and as such it is difficult to ascertain the true divergence in global isolates of this virus. As stated above, several other WSSV-like rod-shaped viruses are known from crustaceans and it may be important to understand the distribution and genotypic similarity of these viruses, particularly when utilising standardised PCR protocols (e.g. from the OIE) in potential new hosts from new locations. Such research may lead to clarification on the current wide host range to WSSV.

Mortalities and importance

The disease is considered to be the most significant disease, in terms of economic loss, to the global aquaculture of shrimp, causing 100% mortalities in few days in culture situations. No effective treatment or vaccines are available.

Other comments

Appraisal of the documents often revealed the lack of information about the circumstances of the isolation (e.g. accurate location, level of mortality, genotyping of virus). An outcome of this review could be a standardised system for describing disease outbreaks in the field and also, a standardised assessment of susceptibility in novel hosts by fulfilment of EFSA criteria.

Yellowhead disease: disease detection, pathogen identification and typing

Review author(s) & Date

Dr Grant Stentiford
Cefas, Weymouth, United Kingdom

October 2009

Causative agent description

Yellowhead Disease is generally considered to be infection with Yellowhead Virus (YHV). YHV (genotype 1 of the yellowhead disease complex) is one of six known genotypes in the complex and is the only known agent of yellowhead disease (i.e. associated with most major outbreaks of disease associated with these viruses). YHV and other genotypes have been classified as a single species in the Genus *Okavirus*, Family *Roniviridae*, Order *Nidovirales*. Virus particles appear rod shaped with a helical nucleocapsid showing parallel cross-striations, prominent surface projections and measure c. 40 x 200nm. YHV is a positive sense ssRNA virus and replicates within the cytoplasm of infected host cells. Gill-associated virus (GAV) is designated as genotype 2 of the Yellowhead complex and occurs with four other known genotypes (genotype 3-6) in healthy *Penaeus monodon* in East Africa, Asia and Australia. Genotypes 2-6 are rarely associated with disease in these regions. Sequencing of ORF1a and ORF1b has aided the discrimination of YHV (genotype 1) from GAV and the other viral genotypes in the complex.

Clinical signs of YHD occur in *P.monodon* within 7-10 days of exposure. YHV has been transmitted horizontally by injection and ingestion of infected material and by immersion in membrane-filtered tissue extracts or by co-habitation with infected shrimp. GAV has been demonstrated to transmit vertically, probably by surface contamination or infection of tissue surrounding the eggs.

Prevalence of viruses within the complex can be high (50-100%) in farmed and wild populations of *P. monodon* in Australia, East Africa and Asia but the prevalence of YHV (genotype 1) in healthy wild or farmed *P.monodon* is generally (<1%), increasing to very high (up to 100%) during Yellowhead disease outbreaks in ponds.

The geographical distribution of the different isolates has been studied. YHV is found in (not exhaustive): People's Republic of China, India, Indonesia, Malaysia, the Philippines, Sri Lanka, Taipei China, Thailand and Vietnam; GAV and other genotypes are found in Australia, India, Indonesia, Malaysia, Mozambique, the Philippines, Taipei China, Thailand and Vietnam.

YHV can induce up to 100% mortality in infected *P.monodon* ponds within 3 days of the first appearance of clinical signs. YHV infected shrimp demonstrate frequent coinfections with bacterial pathogens, these secondary opportunistic pathogens may play a role in the eventual mortality of YHV infected shrimp. Despite being considered as a less pathogenic genotype within the complex, GAV has been associated with mortalities up to 80% in *P.monodon* ponds in Australia. No effective vaccines for viruses within the Yellowhead complex are available.

A comprehensive assessment of host susceptibility to viruses in the Yellowhead complex has recently been completed by EFSA (EFSA 2008). Gulf brown shrimp (*Penaeus aztecus*), Gulf pink shrimp (*P. duorarum*), Kuruma prawn (*P. japonicus*), black tiger shrimp (*P. monodon*), Gulf white shrimp (*P. setiferus*), Pacific blue shrimp (*P. stylirostris*), and Pacific white shrimp (*P. vannamei*) are currently listed as species susceptible to Yellowhead virus complex

genotypes in EC Directive 2006/88. However, the EFSA assessment identified scientific literature to confirm susceptibility in 18 potential host species. Using the EFSA principles of susceptibility, scientific data are available to support susceptibility of *P. monodon, P. merguiensis, P. vannamei, P. setiferus, P. aztecus, P. duorarum, Metapenaeus brevicornis, M. affinis* and *Palaemon styliferus*. Additionally, there are scientific data suggesting susceptibility of *P. esculentus, P. japonicus, P. stylirostris, Metapenaeus ensis,* and *M. bennettae* though some uncertainty exists regarding the identity of the specific pathogen within the complex being assessed. Information on *P. esculentus, M. ensis, M. bennettae,* and *Macrobrachium lanchesteri* was considered insufficient to scientifically assess susceptibility with regards to the EFSA criteria. Scientific data suggesting susceptibility of *M. lanchesteri, M. sintangense,* and *Palaemon serrifer* are essentially experimental and invasive. With the exception of *P. japonicus*, no temperate water species have been specifically tested for susceptibility to viruses in the Yellowhead complex.

Summary of methods findings

Gross signs

Penaeid shrimp. Moribund shrimp may exhibit a bleached overall appearance and a yellowish discoloration of the cephalothorax caused by the underlying yellow hepatopancreas, which may be exceptionally soft when compared with the brown hepatopancreas of normal shrimp. YHV can infect cultured shrimp from late postlarval stages onwards, but mass mortality usually occurs in early to late juvenile stages. High feeding activity may be followed by an abrupt cessation of feeding 2-4 days following the appearance of gross clinical signs of disease and mortality. Moribund shrimp may congregate at pond edges near the surface. In many cases, total crop loss can occur within a few days of the first appearance of shrimp showing gross signs of the disease. External signs are not pathogen specific. Therefore gross observations should not substitute more specific methods for diagnosis. Similarly, gross signs of GAV disease include swimming near the surface and at the pond edges, cessation of feeding, a reddening of body and appendages, and pink to yellow colouration of the gills. However, these signs occur commonly in diseased shrimp are not considered a reliable method for diagnosis of GAV disease.

Crabs, crayfish, freshwater prawns, spiny lobsters and clawed lobsters: Unknown

Clinical Pathology

*Histology***.** Systemic necrosis of ectodermal and mesodermal tissues with prominent nuclear pyknosis and karyorrhexis. Cells contain moderate to large numbers of deeply basophilic, evenly stained, spherical, cytoplasmic inclusions approximately 2µm in diameter or smaller. The lymphoid organ, haemocytes, haemopoietic tissue, gill, heart, cuticular epithelium, midgut and connective tissues are the primary target tissues and organs for YHV infection. Cellular changes in early infections may include nuclear hypertrophy, chromatin diminution and margination, and lateral displacement of the nucleolus. Loss of tissue structure within lymphoid organ, stromal matrix cells that comprise tubules become infected leading to loss of tubular structure, tubules appear degenerate. Lymphoid organ spheroids (LOS) develop during infection, ectopic spheroids may lodge in constricted areas of the haemocoel (heart, gills, subcuticular connective tissues etc). Necrosis of the lymphoid organ in YHD infections can be used to distinguish YHD from acute Taura Syndrome (TS) in penaeid shrimp. Note: the lack of a lymphoid organ in several other host groups within the Decapoda prevent this being used as a target for assessing clinical pathology in these hosts.

Transmission Electron Microscopy (TEM). Nucleocapsid precursors and complete enveloped virions can be observed in YHV infected cells. Nucleocapsid precursors are long filaments approximately 15nm in diameter and of variable length (80-450nm) that occur in the cytoplasm, sometimes enveloped, measuring 40-60nm x 150-200nm with rounded ends and prominent surface projections (8-11nm). Virions can be found in the cytoplasm of infected

cells, in association with intracellular vesicles and can be seen budding at the cytoplasmic membrane and in interstitial spaces. GAV virions and nucleocapsids are indistinguishable from YHV by TEM.

RT-Polymerase Chain Reaction (RT-PCR)

The OIE recommends three RT-PCR protocols. The first is a one-step RT-PCR based on that of Wongteerasupaya *et al*. (1997) used for confirmation of YHV in shrimp from suspected Yellowhead disease outbreaks. This test will not detect GAV or other genotypes in the complex. The second is a more sensitive multiplex nested RT-PCR protocol adapted from Cowley *et al*. (2004) that can be used for differential detection of YHV and GAV in disease outbreaks or for screening. The third (unpublished) protocol is based on a sensitive multiplex RT-nested PCR procedure by Wijegoonawardane, Cowley and Walker that can be used for screening healthy shrimp for viruses in the YHD complex and detects all six currently known genotypes. The test does not however distinguish between the genotypes though this can be achieved by nucleotide sequence analysis of the RT-PCR product. Details of these test protocols are available in the most recent OIE Manual of Diagnostic Tests for Aquatic Animals).

In situ hybridisation (ISH). The suggested protocol is that developed by Tang & Lightner (1999), this method is suitable for detection of YHV and GAV.

Sequencing. Sequencing of the ORF1a and ORF1b regions can be utilised to discriminate YHV (genotype 1) from other viruses in this complex.

Gaps in knowledge

Currently the literature categorises the Yellowhead complex viruses into two distinct viruses: Yellow Head Virus (YHV) and Gill Associated Virus (GAV). GAV is the junior synonym of Lymphoid Organ Virus (LOV). LOV is considered relatively asymptomatic and GAV subsequently reported as its more pathogenic relative, both found strongly associated with the lymphoid organ of *P. monodon*. Via sequencing, LOV and GAV are seen to be 98.9% similar (Cowley et al. 2000), indicating that they are likely the same virus. In addition, GAV had a between 83% and 85.1% similarity to YHV based on sequencing of different amplicons and therefore GAV was reported to be a closely related 'topotype' of YHV. Both of these studies however only used the sequences of relatively few clones (see Cowley et al. 1999). Owing to the small number of clones sequenced and the small size of the sequenced region, the research did not consider the potential variation in mutants within the clones that can constitute so-called quasi-species within a population in addition to possible natural genomic variation within these viruses. Based upon the ICTV definition of a virus species as '…(a) polythetic class of viruses that can constitute a replicating lineage and occupy a particular ecological niche', Van Regenmortel (2000) lists the following characteristics for discriminating between virus species: relatedness of genome sequence, natural host range, cell and tissue tropism, pathogenicity and cytopathology, mode of transmission, physicochemical properties of virions and antigenic properties of virions. Owing to GAV and YHV sharing the above characteristics and with a genome matching 491 out of a compared 577bp, combined with the fact that the viruses are morphologically indistinguishable and cause the same gross disease, Munro and Owens (2007) consider that GAV and YHV are the same virus and term them 'Yellow head-like virus' (YHLV) in their recent review.

Recent work on genetic diversity in the yellow head nidovirus complex lists at least 6 genetic lineages (genotypes). Genotype 1 (YHV) was only detected in *P. monodon* with Yellowhead disease (YHD), Genotype 2 (GAV) was detected in *P. monodon* with less severe condition described as 'mid-crop mortality syndrome' (MCMS) and in healthy shrimp from Australia, Thailand and Vietnam. Other genotypes occurred in healthy (asymptomatic) shrimps. Sequence comparisons of structural protein genes (ORF2 and ORF3), intergenic regions (IGRs) and the long 3'-UTR support the delineation of genotypes and identified conserved

and variant structural features. It is noted that MCMS is far less severe than YHD (less rapid and rarely causing mortalities, less virulent in bioassays). It appears that GAV (genotype 2) is an endemic natural infection of *P. monodon* in eastern Australia and that other genotypes have not been introduced because of prohibition of live crustacean import. Presence of genetically similar GAV isolates in Thailand likely reflect their introduction from import of Australian broodstock. Indian isolates cluster as genotype 4 and Mozambique viruses generally in genotype 6. Genotype 2 (GAV) and 6 appear very similar (a feature also reflected in Australian and African strains of IHHNV in *P. monodon*). Note: recombination of YHD complex isolates also seems to be occurring in Asia at present.

In terms of European legislation, it is important to a. consider the diversity of viruses in the YHD-complex and to assess the relative risk that different isolates pose to European species and b. to assess the efficacy of OIE recognised testing procedures (particularly RT-PCR) for use in novel hosts from novel environments (e.g. temperate waters of the EU). It is recommended that until differentiation of specific isolate effects are demonstrated; diagnostics should follow procedures to detect all viruses within the YHD complex and that all isolates have the potential for negative effects in susceptible species found within European aquatic habitats.

Due to the demonstrable survival of YHV in raw frozen commodity products arising from infected harvests, there is an important gap in knowledge regarding the potential for infectious doses (to different species) within products of different types and furthermore, the potential for exposure to, and consequence for, naïve populations of susceptible species. Furthermore, countries receiving YHV-infected frozen raw commodity products will likely contain infected products in retail outlets and in processing establishments.

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Yellowhead disease: occurrence and distribution

Review author(s) & Date

Dr Grant Stentiford
Cefas, Weymouth, United Kingdom

October 2009

Selection of studies

ASFA and Scopus were databases most frequently used. Search terms included: YHD, YHBV, YHV, GAV, LOV, yellowhead, yellowhead disease, yellowhead virus, yellow head disease, yellowhead virus complex, gill associated virus, lymphoid organ virus. Databases were searched during 2009.

Several reports of YHD were considered to be in "grey" literature (including reports from the OIE/WAHID database). In the latter cases, the evidence, particularly where confirmation of disease agent occurred at an OIE reference laboratory would be taken as "Definite".

Data and analyses

Approximately 200 documents were appraised, and data from approximately 25 were entered into the data table. The aim was to include studies reporting the isolation/detection of the virus, particularly if they reported a new country or new host species. Papers recording the detection of the virus based on PCR alone were in general not included, and definitely not if sequencing was not done. Some of the studies appraised collections of a number of isolates from different sources and then compared them by molecular biology methods. Most often the only information about an isolate was host species, location and year of isolation although all three attributes may not have been reported. Such studies may not have been included unless they added a new host or country, or added data to another publication already in the data table.

The quality ratings applied to the evidence presented for occurrence were:

- **Definite**: full details of identification methods were given or confirmation came in a subsequent paper or confirmation was done in a second laboratory; validated methods used.
- **Probable**: Most details of identification were given; validated methods used.
- **Possible**: Some details of identification given; no confirmation reported.
- **Doubtful**: Scant or no details given and or non validated methods used; no confirmation reported.

Summary of disease findings

Although the causative agent of YHD was named Yellowhead Virus (YHV), the taxonomy of the agent has been the subject of some controversy. Originally reported as baculovirus-like, pathological and molecular evidence showed that in contrast to the DNA-containing baculoviruses, YHV primarily resided in the cytoplasm of infected host cells and was therefore likely an RNA virus. Later studies provisionally classified YHV as a rhabdovirus-like agent. More recent work has shown YHV to be a positive sense ssRNA virus in the genus Okavirus in a new family (Roniviridae) of the order Nidovirales. There is evidence for at least 6 genotypes within the complex with the original clade from Thailand (genotype 1) differing from Gill Associated Virus (GAV) (genotype 2) clade from Australia by approximately 15% of its

nucleic acid sequence. There is evidence of genetic recombination between the genotypes. The disease associated with these viruses (particularly genotype 1) is considered to be one of most significant diseases in Asian shrimp farming, in terms of economic loss, causing up to 100% mortalities in few days in culture situations. No effective treatment or vaccines are available.

Other comments

Appraisal of the documents often revealed the lack of information about the circumstances of the isolation (e.g. accurate location, level of mortality, genotyping of virus). An outcome of this review could be a standardised system for describing disease outbreaks in the field and also, a standardised assessment of susceptibility in novel hosts by fulfilment of EFSA criteria.

6.3. Annex C

Report of quality audit of the disease reviews

Consistent use of the data entry sheet

Inconsistencies were identified both within the same datasheet and between datasheets (i.e. between reviewers).

Mortality. Many papers were investigations of the presence of the pathogen. In general these papers did not report morbidity (fields 5 and 6, stage of disease and signs of disease, respectively) or mortalities (field 7). In these cases most reviewers entered 'not reported' (though some reviewers used a range of synonyms within the same datasheet) or similar. However, some reviewers concluded that no mortality had occurred and entered 'no mortality' or similar (e.g. WSSV datasheet). Therefore, an entry of 'no mortality' cannot be interpreted differently to 'not reported' without reconsideration of the report.
Report type (field 24) and *Classification of report* (field 32). Firstly, the guidance notes did not clearly distinguish these two fields and secondly, many reviewers did not follow the guidance. A number of authors inserted the same text (or very similar) in both fields. Others used field 24 to record the type of publication (e.g. peer-reviewed article, grey literature etc.). Some reviewers limited themselves to a small number of options, e.g. surveillance, research, review (but they are not defined), though in some cases they do not appear to have been consistently applied. In other cases, strings of text has been entered which summarises of the work conducted. Some reviewers left either or both columns empty. Currently, these fields cannot be used to produce summary statistics about the papers in the database.

Quality assessment. Guidance suggested that reviewers use five descriptors: definite, probably, possible, dubious and rejected. These terms were not defined. Some reviewers used these terms others used only three of four of these terms or similar terms (e.g. likely, good). Other reviewers provided strings of text to describe the quality, for example see the table below for text entries used to describe the quality of VHSV papers. Some reviewers made a note that data was not available on which to make an assessment (see table below). If single word descriptors have been used, most reviewers added qualifying text for some if not most citations; as a result these fields will need cleaning before they can be used to produce summary statistics. Only in a small number of datasheets 'rejected' been used to describe quality, and in these case only for a small number of papers. The very low level of 'rejected' papers raises the question about how the screening process was undertaken, and whether other papers were rejected at an early stage. The wide range of descriptors used and the lack of clear definitions rendered the auditing of this field difficult (see following section for conclusions and suggestions for improvement).

Table 1 . Entries in 'quality assessment field for VHSV

Definite
Highly likely
Most likely
Most likely based on sequence data
Definite from sequencing. Antisera for neutralisation had low titre. No mention of use of positive control for neutralisation.
Diagnosis by visual examination can be inaccurate. No data on confirmatory tests in this paper.
Few data on which to make a judgement
Identification only by clinical signs and histopathology. Investigator is very experienced, and the report could be correct, but there was no identification of the disease agent.
Identification uncertain. Unspecified clinical signs were observed and some cases ere reported to have been confirmed by an immunoperoxidase assay using reagents at were not described in the text. The RT-PCR was an in-house assay and no results th p
Likely from serology; newly developed RT-PCR used but no reaction with a positive control VHSV was shown or described, nor sequencing reported
Most likely from the report. This finding was "definite" in a subsequent publication (Smail, D.A. [2000]. Isolation and identification of Viral Haemorrhagic Septicaemia (VHS) viruses from cod Gadus morhua with the ulcus syndrome and from haddock Melanog
Most likely. No details of tests were given in the paper, but the laboratory is the European Community Reference Lab for fish diseases, and so we can have confidence in their results.
No data on which to make a judgement
No details given of the confirmatory tests. Quite likely from IFAT.
No details of results given in this paper, but a probe method that can identify VHSV was used.
No details of test results given in this paper, and so there is no evidence for the conclusions.
No details of test results given in this paper.
No information on typing methodology,
No specific data in the results, but they are most likely accurate
No specific test data in the results as this paper gives the results over a 4-year period. However the methods are comprehensively described in another paper, and are most likely accurate
Very few data are given of virus identification, but fuller details are given in a refereed paper
Very few data are given of virus identification..
Virus was described as being VHSV-like on its biological properties. On the basis of the serology described it does not appear to be typical VHSV.

Keywords: This field was not completed in some of the reviews.

Confirming laboratory: For some reviews (e.g. WSD) the confirming laboratory is given as the reporting laboratory It is debatable whether the confirming laboratory can be the reporting laboratory. It was not clear how 'confirmation' was defined as a separate activity to the initial diagnosis.

Individual audited papers

The key information from all papers audited (e.g. correct identification of the pathogen and its location) was accurately recorded in the datasheets. Suggestions to improve the record of audited papers were passed to the process manager for consideration.

Comparison of geographic occurrence with other data sources

Searches of ProMed by pathogen name indicated that ISAV was more widespread in Chile (outbreaks during 2009) than suggested by the entries in the datasheet (which only used references prior to 2009). An entry on ProMed also provided strong evidence for the presence of *Mikrocytos makini* in the US, and the reviewer decided to make additional entry.
The database produced through literature searches in this project produced different worldwide distributions of some pathogens compared with that derived from the OIE database for aquatic animal diseases (see table below). This finding was discussed with the project manager and process manager.

Table 2. Countries reporting notifiable pathogens to OIE (as recorded on the OIE aquatic animal disease database[1]) not confirmed by reports identified through literature searches

Crustacean pathogens	**Fish pathogens**				**Mollusc pathogens**
WSSV	**EHN**	**KHV**	**VHSV**	**IHNV**	***Marteilia refringens***
Burma	India	Hong Kong	Brazil	Bolivia	Kuwait
El Salvador	Kuwait	Ireland	Estonia	Kuwait	Portugal
Indonesia	Peru	Luxembourg	Ireland	The Netherlands	
Nicaragua		Sri Lanka	Korea	Poland	
Panama			Kuwait		***Bonamia ostreae***
Peru			Kyrgyzstan		Kuwait
Singapore			Laos		***Perkinsus marinus***
Sri Lanka			Latvia		Kuwait
			Lithuania		
			Slovakia		
			Slovenia		
			Sweden		
			Turkey		

Reviewers' comments

The main points made by reviewers were:

- It is not clear where data on prevalence of infection should be recorded

[1] **The database can be found at http://www.collabcen.net/idaad/ and was accessed on October 15, 2009.**

- Many papers only name geographical features, such as rivers or bays; a central location was chosen for recording as the Point Location (field 21).

- The field for wild or farmed was problematic for molluscs; in some studies juveniles reared in hatcheries were released into the wild, while others gathered wild animals and studied them in laboratory/hatchery situations.

- Reference date and date officially reported were confusing as the reference date and the date reported were often the same in the entry table. Date of outbreak or sampling was often used.

- Report type and classification of report were seen as essentially the same.

- Many of the cells could only handle a limited amount of input thus it was not possible to always note the exact words of the papers as suggested in guidance notes.

- Stage of disease was not useful for molluscan pathogens (for *Marteilia* and *Perkinsus* it was used to indicate the stages observed in the parasite lifecycle)

- It would have been easier if adjacent field were available for mortality, prevalence and population size

Deriving information from the database

The key guidance to reviewers was for reviewers to use the text provided in the papers reviewed as far as possible, including typographical errors etc. This is not relevant for the fields that required interpretation by the reviewer (e.g. report type, classification, quality assessment etc.). The database as it stands fulfils its primary purpose of describing the geographic location of the selected pathogens. Some work will be required to standardise country names. Additionally, summary information for each paper can be quickly retrieved.
The combination of the datasheets in a single database could allow more in depth analysis of the data; for example, the following questions could be addressed:

- For each pathogen, what was the average and range of mortality reported in outbreak investigations? Does this vary between countries?

- For each pathogen, what was the average and range of prevalence of infection reported in surveys? Does this vary between countries?

- How does the quality assessment of the reports vary between pathogens and country?

Currently, the format of the data does not allow for simple cross-tabulations required to answer the questions above, in part because the data entry was not controlled for either report type or mortality fields. Secondly, prevalence and mortality data is not currently stored in a numeric format necessary for calculation of averages etc.. In addition, the lack of consistency between reviewers in how these parameters were recorded (see comments above) does not at the moment allow for analysis using data from more than one pathogen. The necessary information has been recorded, and could be extracted into a usable format without referring back to the original source (but it is unlikely that this could be done automatically). Notwithstanding some inconsistent use of controlled entries within a single review (which could be easily resolved), summary statistics by pathogen can be derived for some fields for only a few pathogens. For example, there are 64 citations reviewed for *Bonamia* species *(Bonamia ostreae* and *Bonamia exiotosa)* (yielding a total of 107 entries); only 4 descriptors were used for the quality assessment allowing for the analysis in the table below.

Table 3. Breakdown of *Bonamia ostreae* and *Bonamia exiotosa* citations (n=64) by quality assessment

Definite	Probable	Possible	Rejected
21% (14)	24% (16)	45% (30)	9% (6)

(number of citations)

Quality assessment

It is highly likely that the analysis discussed in the section above would need to take account of the quality assessment (field 33). Firstly, rejected would need to excluded from analysis, additionally, it may be desirable in some cases to analyse only definite records. To this end the entries in this field need to be standardised, as far as possible, using the definitions developed each reviewer (who have provided their working definitions in the summary reports). Where text strings have been entered these need to be replaced with a single word descriptor (the text can be preserved in an additional field).
The starting point for the quality assessment is that the paper concludes that a pathogen or a disease is present. It was clear that the quality assessment was made primarily on the type and number of diagnostic tests used. On this basis definitions for quality descriptors can be developed. It is clear from the table above, listing the quality descriptors for VHSV papers, that two columns to assess quality are needed: one for the entry of a single word descriptor (controlled) and a second column to record qualifying comments (free text).

Classification of reports

Classification of the reports allows a user of the database to deduce whether the paper refers to an investigation of a disease outbreak (mortality and morbidity recorded) or surveillance for the pathogen (in populations where clinical disease has not necessarily been reported). Most papers fall into one or other of these two broad categories. It is suggested that in future this information is captured in a single field. The primary purpose of the database is to map the distribution of the selected pathogens; however, information on the occurrence of disease is also useful (and may allow hypotheses to be generated about different expression of the pathogen, e.g. level or mortality, morbidity, in different species or environments). Other fields should be provided to allow additional information about the report to be captured. Limited options should be provided to cover most types of reports with a final 'other' option to be complemented with free text describing the report. This will allow summary statistics to be generated about the reports in the database. Fields could include i) peer-reviewed / non peer-reviewed, ii) experimental investigation / field work / surveillance / outbreak investigation.

Comparison with other data sources

The presence of a number of pathogens has been reported to OIE, but supporting publications in the literature have not appeared hence there are discrepancies in the worldwide distribution depending on the sources of data.

Concluding comments

The overall conclusion of the audit is that an accurate record of the papers reviewed has been achieved. The database thus provides a useful summary of the relevant papers on distribution of the selected pathogens. The need to record the information exactly as provided in each paper does not make the data in it current format amendable for more in depth analysis (as discussed above). For example, the records of mortality and morbidity are complete and accurately reflect the detail provided in the paper; but further work is needed to extract data into numeric fields for further analysis. Fields to record the reviewers' interpretation of the

paper have not been consistently completed and further work is required before they can be used.

Suggestions are made below for future development of the current database or future data review exercises. The suggested developments will allow more in depth analysis of the data as discussed above.

- Fields for mortality and prevalence are provided (formatted for percentages), and a separate field to record total population (number format).

- A quality assessment field allows only controlled entries (5 pre-defined descriptors); a second field allows qualifying text.

- Descriptors of quality are discussed and defined after discussion with reviewers. Generic definitions may need to be developed in detail for each pathogen (to include named diagnostic tests).

- A number of fields with controlled entries (from a drop down box) are used to capture a description of the paper. Reviewers could use, for example, up to 4 descriptors, to describe the paper.

- The field describing the population as ‘wild’ or ‘farmed’ needs to be extended to account for other possibilities. The origin of animals as wild or farmed needs to be distinguished, in a separate field from where the animals are found when sampled for the survey in question.

6.4. Annex D

Critical review of VHSV genotyping

Issues with viral haemorrhagic septicaemia virus genotyping

It is now generally accepted there are four genetically discrete genogroups (I -IV) of VHSV (Einer-Jensen *et al.* 2004, Elsayed *et al.* 2006, Nishizawa *et al.* 2006, Raja-Halli *et al.* 2006, Stone *et al.* 2008, Dale *et al.* 2009). Genogroup I includes viruses originating in freshwater farm, sites, marine isolates from the Baltic Sea, Skaggerak and Kattegat, North Sea and English Channel; the brackish waters of the Gulf of Finland, and the Black Sea (Raja-Halli *et al*. 2006 Nishizawa *et al.* 2006). Genogroup II consists of the marine isolates from the Gotland Basin (Baltic Sea); Genogroup III includes the isolates from the North Sea coastal waters of the UK and Ireland (Einer-Jensen *et al*. 2004), while Genogroup IV consists of viruses from the Pacific coast of North America and Japan and viruses from the Great Lakes region of North America. Unfortunately however, several different regions of the VHSV genome and a range of different isolates have been used for VHSV typing, and the typing scheme has undergone several changes over the past decade, and consequently it can be difficult to determine if the genotype attributed to an individual VHSV isolate in one of the early publications is still valid. For example, isolate KHRV9601 from the Japanese flounder (*Paralichythys olivaceous*) was assigned to genogroup III using the typing scheme of Stone *et al.* 1997 (Nishizawa *et al*. 2002) whereas it was assigned to genogroup I when using the typing scheme of Einer-Jensen *et al* 2004 (Elsayed *et al.* 2006). Similarly, isolate L59x from eel (*Anguilla anguilla*) was assigned to genogroup II using the typing scheme of Benmansour *et al* (Thiéry *et al*. 2002) whereas it was assigned to genogroup III using the typing scheme of Einer-Jensen *et al*. 2004 (Einer-Jensen *et al.* 2004, Nishizawa *et al.*, Elsayed *et al*. 2006, Raja-Halli *et al.* 2006, Stone *et al*. 2008).

This review will provide an historical overview of the VHSV typing schemes and identify the most appropriate scheme to use in future analyses, and will also review the evidence for the sub typing of isolates within genogroups I and IV.

Historical overview

In 1993, it was demonstrated using hybridisation that the North American and European isolates formed two genetically distinct strains of the virus, in which, isolates from different years or species on each continent were more closely related to each other, than to isolates from the other continent (Batts *et al.* 1993).
These findings were confirmed by phylogenetic analysis based on the complete glycoprotein (G) gene sequences of European and North American isolates (Basurco *et al*. 1995).

In 1997, Stone *et al.* exploited the availability of G-gene sequence to design primers to amplify and sequence a partial G-gene sequence (nt 361- 720) for a range of VHSV isolates from Europe and North America (Stone *et al.* 1997) and showed that there were three distinct genogroups of VHSV (I-II). North America isolates were assigned to genogroup I, the isolates from mainland Europe were assigned to group III and a small group of Isolates from marine fish species from the North Atlantic were assigned to genogroup II. Nishizawa *et al*. (2002) adopted the same typing scheme when looking at the genetic relatedness among Japanese, North American and European isolates of VHSV. They established that there were two genotypes of VHSV in Japan. The first represented by Obama25 was closely related to the North American isolates and were assigned to genogroup I while the other, the KRRV9601 isolate, was shown be closely related to the traditional European isolates and was assigned to genogroup III.

In the same year that Stone *et al.* (1997) identified three genogroups of VHSV virus researchers at the Institute National de la Recherche Agronomique, France also identified three distinct genetic lineages based on partial G-gene sequences (nt 220-1294) but unfortunately they used a different typing system and assigned the traditional European isolates to genogroup I and the North American isolates to genotype III (Benmansour *et al*. 1997). This typing system was then adopted by another French group from the Agence Francaise de Sécurité Sanitaire des Aliments, France (Thiéry *et al*. 2002). In the latter publication they also suggested that the genogroup I could be subdivided in to four subgroups (Ia –Id).

In 1999, further complications were introduced by the work of Snow *et al.* (1999). Phylogenetic analysis based on partial nucleoprotein (N) gene sequences identified a fourth distinct lineage of VHSV from the Baltic sea, but rather than adopting one of the two earlier typing schemes and assigning the new isolates to a genogroup IV, a further change was made to the numbering system for the different lineages. The traditional European isolates were assigned to genogroup I, the Baltic sea isolates were assigned to genogroup II, the North Atlantic isolates to genogroup III and the North American isolates to gengroup IV.

In 2004, both Snow *et al*. and Einer Jensen *et al*. adopted the same typing scheme when analysing partial N gene sequences and complete G–gene sequences respectively (Snow *et al*. 2004, Einer Jensen *et al*. 2004). In the latter, the genogroup I viruses were subdivided in to genogroups Ia to Ie but unfortunately the validity of the sub-typing scheme and the relationship between this subtyping scheme and that proposed by Thiéry et al. (2002) was not adequately addressed and as a consequence viruses assigned to genogroup Ia and Ib by Thiéry were assigned to genogroup Ia. Some isolates (e.g. 14-58 and 23-75) assigned to genogroup Ic by Thiéry were also reassigned to Ia and others (e.g. Hedadam 70 and F1) were left unassigned. It is also not clear if the genogroup Id described by Thiéry *et al.* (2002) is the same as that described by Einer Jensen *et al*. (2004). Snow *et al.* (2004) identified two lineages within genogroup I. These appear to be consistent with the genogroups Ia and Ib described by Einer-Jensen *et al.* (2004), but again, other viruses were not unassigned to a subgroup and many of the viruses analysed by Einer-Jensen *et al.* (2004) were not included in the study.

In 2006, Nishizawa *et al.* expanded their phylogenetic analysis to include additional isolates from the Black sea (Nishizawa *et al*. 2006). The neighbour joining tree based on nt 360-720, which included sequence data from Einer-Jensen *et al*. (2004), identified the four main genogroups (I-IV) and subgroups (a-e) within genogroup I. The labelling of the genogroup and sub genogroups was consistent with the typing scheme described by Einer Jensen *et al.* (2004) with the exception that the previously unassigned isolates, Hededam, 14-58 and 23-75 were placed in subgroup Ia, and DK-F1 was assigned to genogroup Id.

In 2007 Ruszczyk *et al.* undertook a phylogenetic analysis of Polish strains of VHSV based on a partial G-gene sequence (nt 715–995). The analysis, which included sequence data from more recent publications, identified the four major genogroups and these were labelled according to the scheme described by Einer-Jensen *et al.* (2004). The Polish isolates were assigned to genogroup I (Ruszczyk *et al.* 2007*).*

Following an outbreak of VHS in sea-water farmed rainbow trout in Norway in 2007 the infectious agent was initially confirmed by RT-PCR based on a partial G-gene sequence. Typing was undertaken based on the complete G-gene sequence and using the scheme proposed by Einer-Jensen *et al.* (2004) (Dale *et al.* 2009).

Analysis of the complete G gene of a VHSV isolate recovered from muskellunge *Esox masiquinongy* in Lake Claire in 2003 revealed a new sub lineage (genogroup IVb) of the North American genotype (Elsayed *et al*. 2006). Further isolates from a range of fish species

from the Great lakes region of the US were also assigned to genogroup IVb based on complete G-gene sequences (Winton *et al*. 2007).
In 2007, Lumsden *et al.* also included sequence data from Einer-Jensen *et al*. (2004). Performing neighbour-joining analyses based on partial G-gene sequences (nt 360-720) they adopted the same typing scheme as Einer-Jensen to show that the VHSV isolated from freshwater drum in Canada was similar to the isolates assigned to genogroup IVb by Elsayed *et al*. 2006 (Lumsden *et al*. 2007). The presence of genogroup IVb viruses was confirmed in eastern Canada by Gagné *et al.* in 2007 using partial G gene sequence using isolates collected in 2000, 2003 and 2004 (Gagné *et al.* 2007*).*

In addition to the analyses performed above, there is a significant quantity of partial sequence data on the EMBL and GenBank database that remains unpublished.

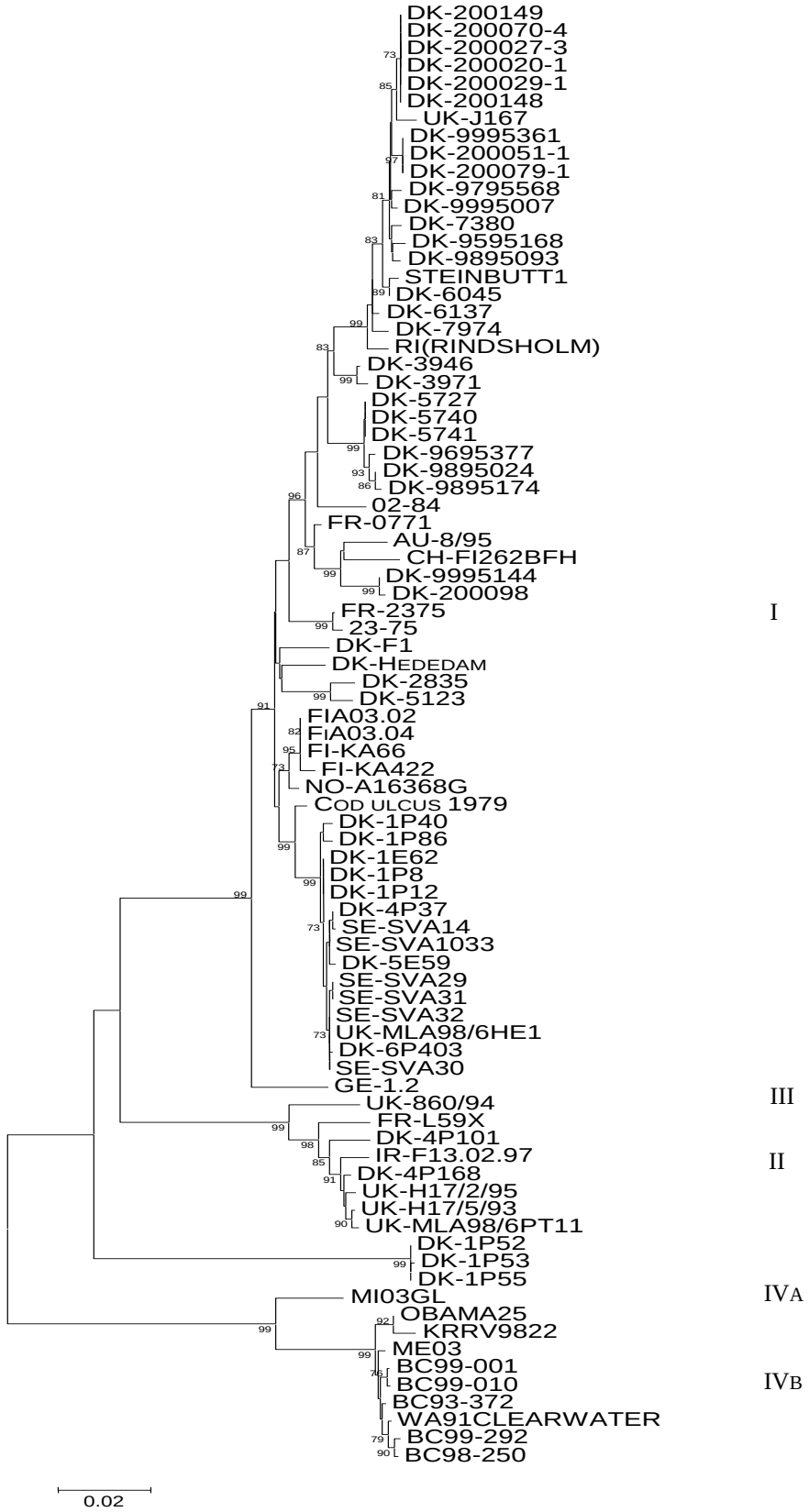

Figure 1. Currently accepted typing scheme for VHSV based on the complete G-gene sequence. Neighbour-joining tree based on complete G-gene sequences taken from Einer-Jensen *et al.* 2004, Elsayed *et al.* 2006, Raja-Halli *et al.* 2006 and Stone *et al.* 2008. The four recognised genogroups are highlighted and named according to Einer-Jensen *et al.* 2004. The potential subdivisions within genogroup I are not shown. Analysis was done on 1000 bootstrapped data sets and values of >70% are shown on the tree. The scale bar represents substitutions per nucleotide site.

Assignment of historical isolates to the genogroups described in the typing scheme of Einer-Jensen *et al.* (2004).

In 2005, Einer-Jensen showed in parallel phylogenetic analyses based on a small fixed group of VHSV isolates that the main genetic lineages identified were the same irrespective of the gene or gene region analysed (Einer Jensen *et al.* 2005). In the following phylogenetic trees the complete G gene sequences were taken from all of the most recent publications (Einer-Jensen *et al.* 2004, Elsayed *et al.* 2006, Raja-Halli *et al.* 2006, Stone *et al.* 2008), truncated to the appropriate size and then combined with the historical sequence data prior to reanalysis using a neighbour-joining method. This allows the two typing schemes to be compared and the reassignment of the historical isolates to a genogroup using the newer typing scheme. Multiple alignments were performed using Clustal W and the phylogenetic analyses were conducted using Neighbour-joining methods using MEGA version 3.1 (Kumar, Tamura & Nei, 2004).

Nucleotides 360 –320 (Stone *et al*. 1997, Nishizawa *et al*. 2002, Nishizawa *et al*. 2006, Lumsden et *al.* 2007)

The neighbour–joining tree based on the truncated sequences (nucleotides 360-620) produced the same main lineages observed when the analysis was performed using the complete G-gene, and the bootstrap analysis provided good support for the four main genogroups I-IV (figure 2). Resolution and bootstrap support of the sub lineages within genogroup I proposed by Einer Jensen *et al*. (2004) was poor and it was not possible to assign a virus isolates to a subgroup. All isolates previously assigned to genogroup I by Stone al 1997 were assigned to genogroup IV, those previously assigned to genogroup II were assigned to genogroup III and those previously assigned to genogroup III were assigned to genogroup I. Isolates from the new genogroup II were not included in the original study. Similarly, all isolates assigned to genogroups I, II and III by Nishizawa *et al.* 2002 were assigned to genogroups IV, III and I respectively.
Trees produced by Nishizawa *et al*. (2006) and Lumsden et *al.* (2007) using nucleotides 360-720 of the G-gene were labelled according to the typing scheme of Einer-Jensen *et al.* (2004). Sequences from these publications were also included in the analysis (figure 2).

Nucleotides 710 – 995 (Thiéry *et al*. 2002)

The neighbour–joining tree based on the truncated sequences (nucleotides 710-995) produced the same main lineages as the trees generated using the complete G-gene, and the bootstrap analysis provided good support for the four main genogroups I-IV (figure 3). Clustering of isolates was consistent with the tree generated by Thiéry et *al. (*2002), but again, the resolution and bootstrap support of the sub lineages within genogroup I proposed by Einer Jensen *et al.* (2004) was poor and it was not possible to assign a virus isolates to a subgroup. All isolates previously assigned to genogroup I by Thiéry et al (2002) were assigned to genogroup I, those previously assigned to genogroup II were assigned to genogroup III, and those previously assigned to genogroup III were assigned to genogroup IV. Isolates from the genogroup II defined by Einer-Jensen *et al.* (2004) were not included in the original study.

Nucleotides 220- 1295 (Benmansour *et al*. 1997)

The neighbour–joining tree based on the truncated sequences (nucleotides 220-1295) have the same main lineages as the trees produced using the complete G-gene, and the bootstrap analysis provides good support for the four main genogroups I-IV (figure 4). Clustering of isolates was consistent with the tree generated by Benmansour *et al.* (1997), but again, the resolution and bootstrap support of the sub lineages within genogroup I proposed by Einer Jensen et al 2004 was relatively poor and it was

not possible to assign a virus isolates to a subgroup. All isolates previously assigned to genogroup I by Benmansour et al 1997 were assigned to genogroup I, those previously assigned to genogroup II were assigned to genogroup III, and those previously assigned to genogroup III were assigned to genogroup IV. Isolates from the genogroup II defined by Einer-Jensen et al 2004 were not included in the original study.

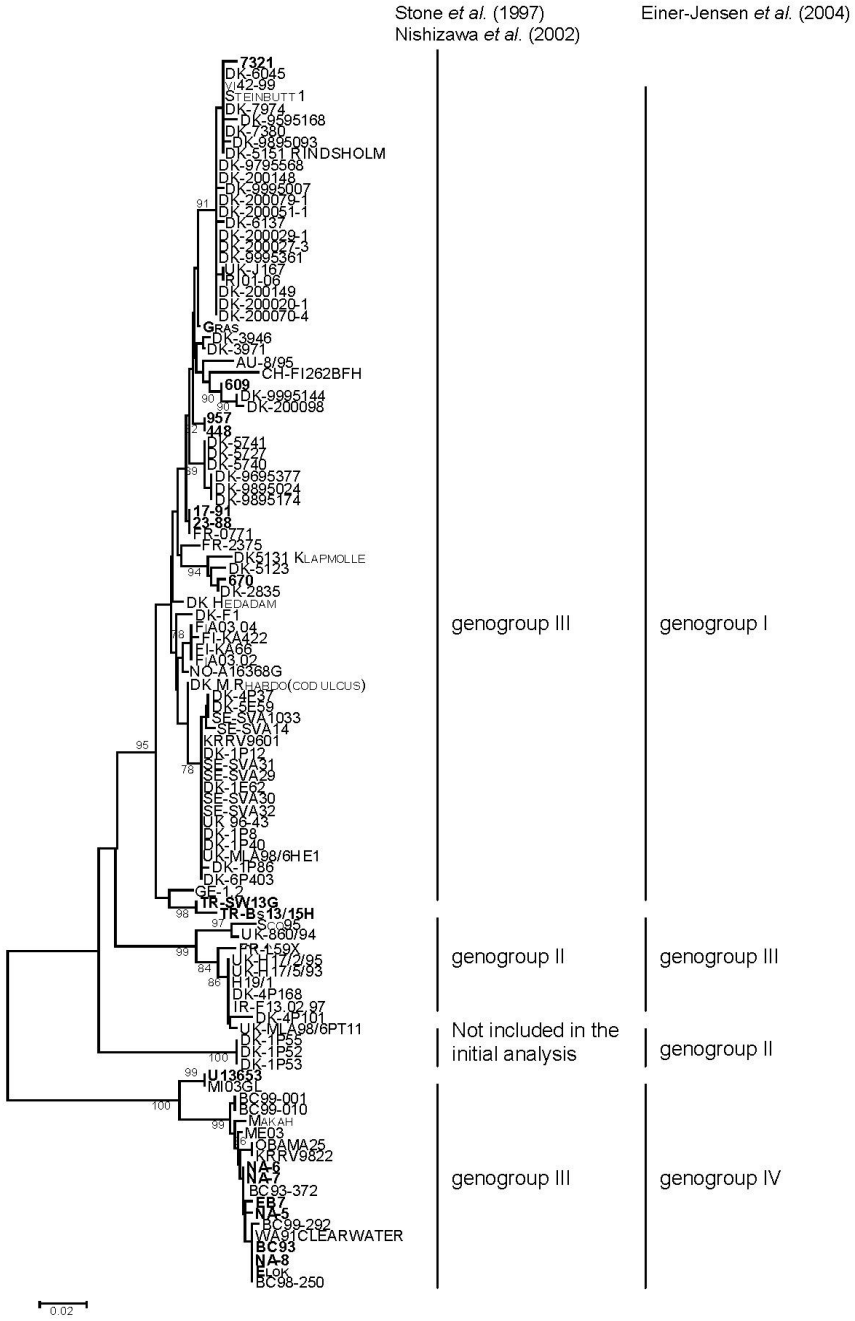

Figure 2. Neighbour joining tree based on partial G gene sequences (nt 360-720) showing the relationship between the typing scheme of Stone *et al.* 1997 and that of Einer-Jensen *et a.l* 2004. Isolates that were not previously assigned to a genogroup based on the complete G gene typing scheme of Einer-Jensen *et al.* (2004) are shown in bold. Isolate U13653 was taken from Lumsden *et al.* 2007 Analysis was done on 1000 bootstrapped data sets and values of >70% are shown on the tree. The scale bar represents substitutions per nucleotide site.

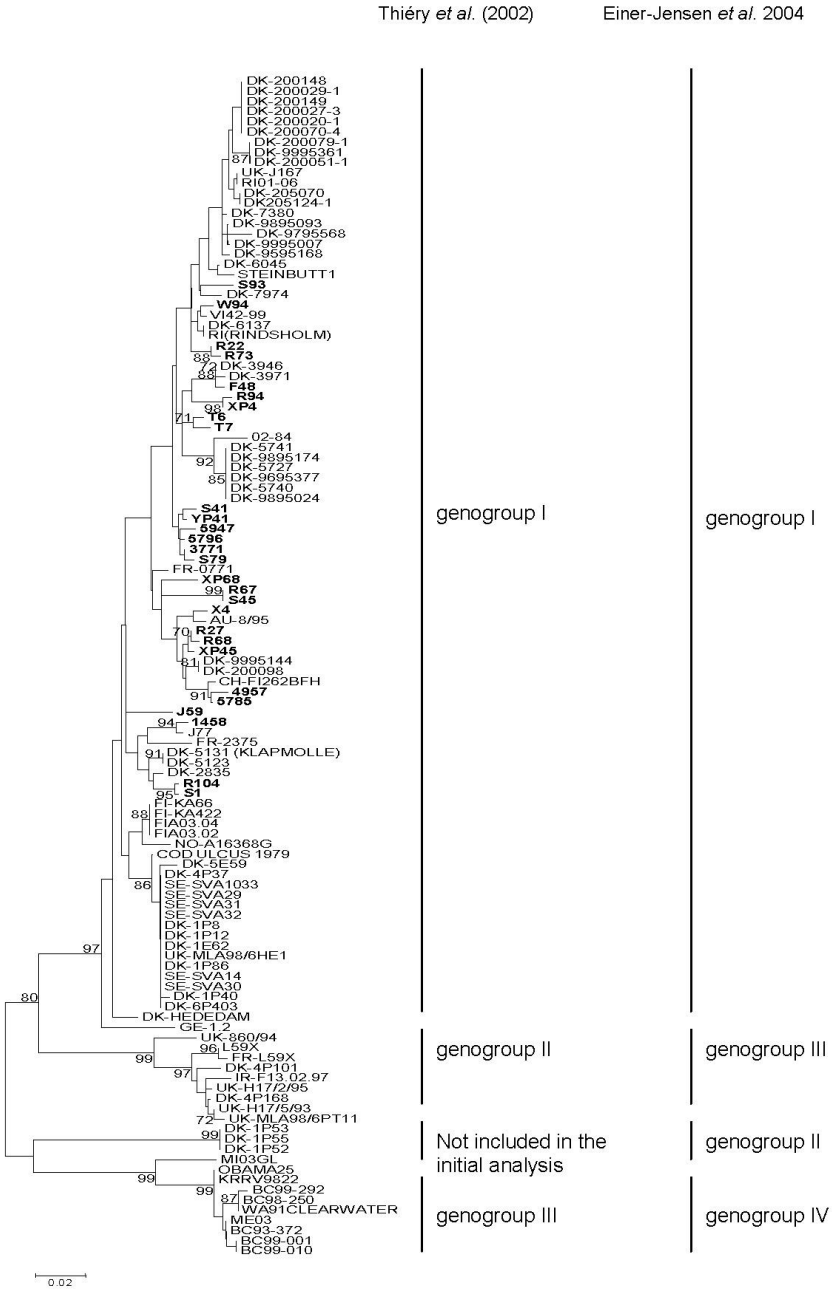

Figure 3. Neighbour joining tree based on partial G gene sequences (nt 710-995) showing the relationship between the typing scheme of Thiéry *et al.* (2002) and that of Einer-Jensen *et al.* 2004. Isolates that were not previously assigned to a genogroup based on the complete G gene typing scheme of Einer-Jensen *et al.* (2004) are shown in bold. Analysis was done on 1000 bootstrapped data sets and values of >70% are shown on the tree. The scale bar represents substitutions per nucleotide site.

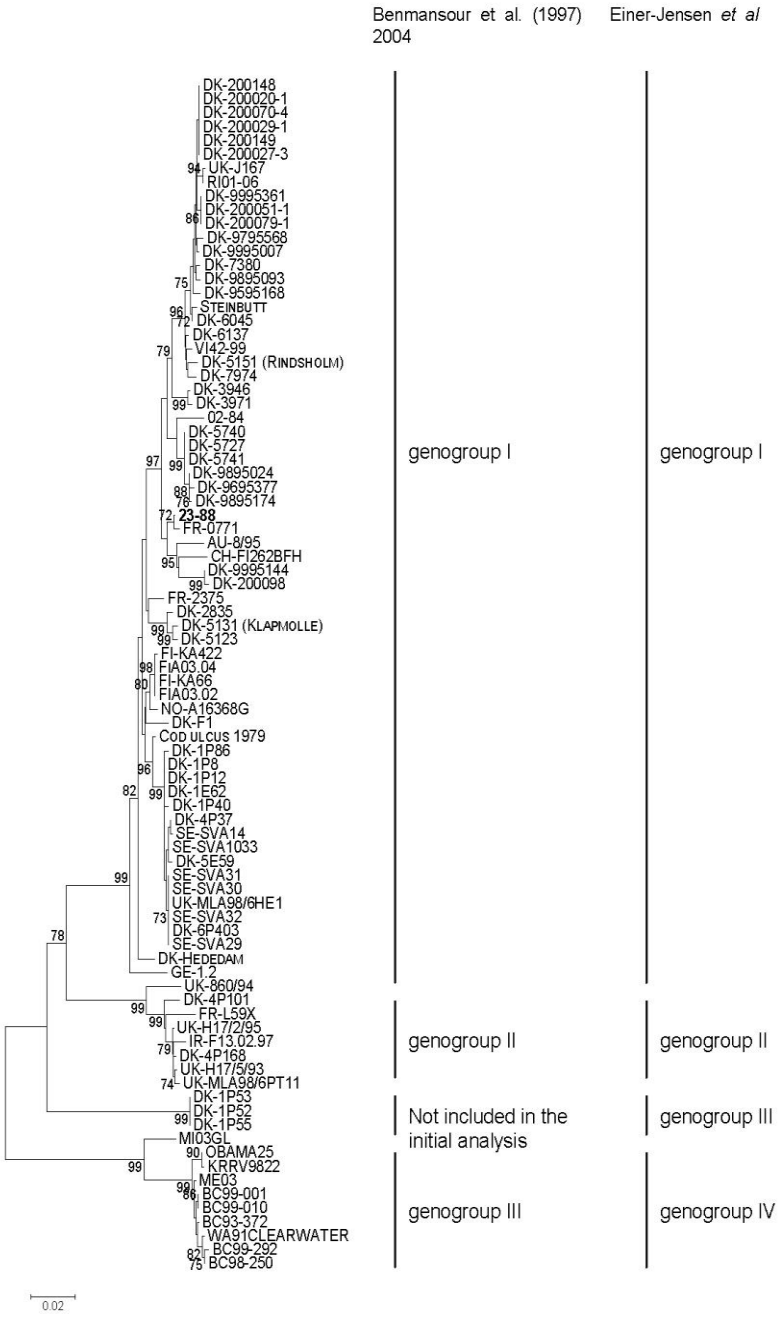

Figure 4. Neighbour joining tree based on partial G gene sequences (nt 220-1294) showing the relationship between the typing scheme of Benmansour *et al.* (1997) and that of Einer-Jensen *et a.l* (2004). Isolates that were not previously assigned to a genogroup based on the complete G gene typing scheme of Einer-Jensen *et al.* (2004) are shown in bold. Analysis was done on 1000 bootstrapped data sets and values of >70% are shown on the tree. The scale bar represents substitutions per nucleotide site.

3050

Conclusions

It is now generally accepted that analysis based on complete G gene sequences is the most robust method for typing VHSV isolates (figure 1), and this approach was used to type the viruses associated with the most recent VHS outbreaks in the US, (Elsayed *et al.* 2006), Finland (Raja-Halli *et al.* 2006) the United Kingdom (Stone *et al.* 2008) and Norway (Dale *et al.* 2009). There is good evidence, supported by bootstrap analysis, for the subdivision of genogroup IV, but it is not clear if the current subdivisions proposed within genogroup I are sufficiently robust. This is because several viruses remain assigned using the typing scheme proposed (Einer Jensen *et al*. 2004), but more importantly, support for the subdivisions as evidenced by bootstrapping, is heavily dependant on the number and range of isolates, and the region of the genome used in the analysis. This will need to be addressed as the number of available complete G gene sequences increases, but in the meantime, it is suggested by the reviewer that, with the exception of sub genogroups IVa and b, the major genogroups are not subdivided. To prevent any further confusion when typing VHSV isolates in the future it is also suggested that any new VHSV genogroup(s) are numbered V, VI, VII etc., irrespective of their genetic relationship to the currently recognised genetic lineages.

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6.5. Annex E

Critical review of identification of *Bonamia* species

Issues on pathogen identification and strain differentiation of Bonamia species.

Four species are currently distinguished in the genus *Bonamia*: ***Bonamia ostreae***, ***Bonamia exitiosa***, ***Bonamia roughleyi*** and ***Bonamia perspora***. Two of the species, *B. ostreae* and *B. exitiosa*, are listed in the EU Council Directive 2006/88/EC. In this review a number of issues on the identification of *Bonamia*-species are discussed below.

Bonamia ostreae

Bonamia ostreae is the type species of the genus *Bonamia* and was described by Pichot et al (1980), mainly based on ultrastructure. The parasite is an intracellular protozoan with as natural host the European flat oyster *Ostrea edulis* (Table 1). No specific study has been carried out on the (molecular) intra-species variation of *B. ostreae*. In the study of Carnegie et al. (2000) the *B. ostreae* 18S-ITS 1 rDNA sequence from 4 oysters (1 Gun Point Creek, USA, 3 Cork Harbour, Ireland) diverged by 0.39% over the 1945 bp. The GenBank sequence from this study (AF262995) and the 18S SSU rDNA sequence in the publication of Cochennec et al. (2000); AF192759) differs at 3 positions over the total of 1271 bp. Hence, the variability between different "isolates" of *B. ostreae* is considered limited and currently no strains of *B. ostreae* are identified.

Bonamia exitiosa

In 1987, a second *Bonamia* species was described in *Ostrea chilensis* on the southern hemisphere (Dinamani et al. 1987). The species was first designated as *Bonamia exitiosus* in 2001 (Hine et al. 2001) and later renamed as *Bonamia exitiosa* (Berthe & Hine 2003). Characterisation of the species was based on ultrastructure and molecular data.

The identity of *B. exitiosa* is currently contentious as there is *B. exitiosa (sensu stricto),* which occurs in *Ostrea angasi* in Australia (Corbeil et al. 2006) and *O. chilensis* in New Zealand (Hine et al. 2001, Berthe & Hine 2004), and other *B. exitiosa*-like organisms that have been reported from *O. chilensis* in Chile (Campalans et al. 2000, Lohrmann et al. 2009), *Ostrea puelchana* in Argentina (Kroeck & Montes 2005), *Crassostrea ariakensis* in the eastern United States (Burreson et al. 2004, Bishop et al. 2006), *O. edulis* on the Atlantic coast of Spain (Abollo et al. 2008) and in *O. edulis* in the Mediterranean. These will be referred to as *B. exitiosa (sensu lato).* The difficulty in assigning isolates to a species is shown by the isolates from Chile which may be identified as *B. exitiosa (sensu stricto),* a sub-species of *B. exitiosa (B. exitiosa sensu lato)*, or another *Bonamia* species (Lohrmann et al. 2009). The ultrastructure of Argentinean, Atlantic Spanish and Adriatic isolates suggests that they are the same species similar to *B. exitiosa* (Hine, Kroeck and Villalba: *unpub. obs*.). The isolates of *B. exitiosa* from Morehead City Bay, North Carolina (Bishop et al. 2006) resemble *B. exitiosa* from Australia, and are possibly a shipping introduction. As the initial mortalities in San Antonio Este, Argentina, occurred in a bed < 1 km from an international shipping wharf, it could also originate from a shipping introduction. The true identity of Chilean, Argentinean, Spanish and Mediterranean isolates must wait on sequencing of more than one gene.

Bonamia roughleyi

The microcell observed in *Saccostrea glomerata* originating from New South Wales, Australia was described as *Mikrocytos roughleyi* by Farley et al. (1988) In the publication of Cochennec-Laureau et al. (2003) the species was subsequently transferred to the genus *Bonamia* based molecular data. The limited molecular work carried out on this species showed a very close relationship with the *B. exitiosa*-like group (Cochennec-Laureau et al. 2003). On the other hand, *Mikrocytos roughleyi* can be distinguished from *B. exitiosa* based

on characteristics in histopathology, ultrastructure and epizootic data (pers. comm. Mike Hine). However, ultrastructural observations were based on poorly fixed material, and further observations are needed. The contrasting description between the ultrastructure and molecular data poses for the moment an unresolvable problem. Further work on this species should be carried out to confirm the results and the placement of *Mikrocytos roughleyi* in the genus *Bonamia*. Hence, *Bonamia roughleyi* was not considered further in this review and analysis on strain identification below.

Bonamia perspora

Bonamia perspora has recently been described as parasite of the oyster *Ostreola equestris* based on histopathology, electron microscopy and molecular phylogenetic analysis (Carnegie et al. 2006). It is currently the only known species in the genus *Bonamia* shown to produce typical haplosporidian spores. Phylogenetic analysis identified *B. perspora* as a sister species of *B. ostreae*.

Table 1 *Bonamia* species from different geographical locations, their (natural) host and description

Agent	(Natural) host	Location	Species description
Bonamia ostreae	*Ostrea edulis*	France	(Pichot et al. 1980)
Bonamia exitiosa	*Ostrea chilensis*	New Zealand	(Hine et al. 2001)
Bonamia exitiosa	*Ostrea angasi*	Australia	(Corbeil et al. 2006)
Bonamia exitiosa	*Ostrea edulis*	Spain	(Abollo et al. 2008)
Bonamia sp.	*Ostrea chilensis*	Chile	(Lormann et al. 2009)
Bonamia sp.	*Crassostrea ariakensis*	North Carolina, USA	(Burreson et al. 2004)
Bonamia sp.	*Ostrea peulchana*	Argentina	(Kroeck & Montes 2005)
Bonamia perspora	*Ostreola equestris*	North Carolina, USA	(Carnegie et al. 2006)

Ultrastructure of Bonamia species

The ultrastructure of *Bonamia ostreae* (Pichot et al. 1980), *B.exitiosa* (Hine et al. 2001) and *B. perspora* (Carnegie et al. 2006) have been adequately described, but the description of *B. roughleyi* (Cochennec-Laureau et al. 2003) is based on few observations of poorly fixed material, and as stated above will therefore not be included.

Bonamia ostreae and *B. exitiosa* are only known from vegetative stages and spores have not been observed despite many years of study. Only *B. perspora* is known to form spores. Care must be taken to compare equivalent stages as all three species have simple uni-nucleate stages that become bi-nucleate and divide into further uni-nucleate stages, but *B. exitiosa* has a large amoeboid trophic stage. The simple uni-nucleate stage of *B. ostreae* is smaller (2.4 ± 0.5 µm) than *B. exitiosa* (3.0 ± 0.3 µm). *B. ostreae* has fewer and larger haplosporosomes (7 ± 5, 153 ± 18 nm) than *B. exitiosa* (14 ± 6, 148 ± 11 nm) and *B. ostreae* has fewer mitochondrial sections (2 ± 1) than *B. exitiosa* (3 ± 1).

In New Zealand, *B. exitiosa* has a well-defined annual pattern of infection (Hine 1991), being found at very low levels in winter, with numerous small uni-nucleate stages being present in spring to early summer, larger amoeboid uni-nucleate stages occurring in late summer and autumn, with the population crashing in early winter. Although the developmental pattern in other oyster hosts is unknown, the large uni-nucleate form, which doesn't occur in *Bonamia ostreae*, has been observed in Australian and Chilean oysters (Lohrmann et al. 2009). Unpublished observations (M. Hine) on the ultrastructure of *B. exitiosa*-like species. from Argentina and Spain suggest that they are identical.

Molecular phylogeny of Bonamia species

Although the majority of the *Bonamia* species lack the characteristic spore stage of haplosporidians, molecular data based on the 18S-ITS1 ribosomal DNA sequence of *B. ostreae* supported the inclusion of *Bonamia ostreae* in the phylum Haplosporidia (Carnegie et al. 2000). Further studies on the placement of the genus *Bonamia* show the *Bonamia* species to form a monophyletic clade (Reece et al. 2004). Within the genus *Bonamia* two separate clusters can be distinguished: a cluster with *Bonamia perspora* and *Bonamia ostreae* and a cluster of *Bonamia exitiosa*(-like) microcells (Fig. 1).

Most of the sequence data available for phylogenetic analysis of the family Haplosporidia is restricted to two sites: the ribosomal RNA and actin gene sequences. For the genus *Bonamia* the actin gene sequences are only known of *Bonamia ostreae* (Lopez-Flores et al. 2007). Two actin genes were identified and subsequent phylogenetic analysis place *Bonamia ostreae* in a clade with *Minchinia* species, which is reflected in similarity in ultrastructure (Hine et al. 2009).

A number of unpublished sequences of the ribosomal RNA gene complex of *Bonamia* species is available from GenBank, submitted by researchers of the Virginia Institute of Marine Science (Gloucester Point, VA, USA). The data consists of 497-550 bp fragments of 18 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence. This fragment has been sequenced from different clones of *Bonamia ostreae* (USA), *Bonamia exitiosa* (New Zealand), *Bonamia perspora*, *Bonamia* sp. from *Crassostrea ariakensis*, *Bonamia* sp. from *Ostrea chilensis* (Chile), *Bonamia* sp. from *Ostrea angasi* (Australia) and *Bonamia* sp. from *Ostrea puelchana* (Argentinia). The origin has not been indicated for all clones. In figure 2 a phylogenetic analysis is shown of the available 18S-ITS1-5.8S-ITS2 sequences (shortest fragment 497 bp in length). These preliminary analysis indicate a very high sequence similarity especially between the clones of *Bonamia exitiosa*, *Bonamia* sp. from *O. puelchana* and *Bonamia* sp. from *C. ariakensis*. Figure 3 shows the phylogenetic analysis of the available 18S-ITS1-5.8S-ITS2 sequences including *O. angasi* (shortest fragment 345 bp in length). The sequences of *Bonamia* sp. in *O. angasi* cluster together with the *Bonamia* species originating form *O. puelchana* and *Crassostrea ariakensis*. From these preliminary analysis the differences between *Bonamia* sp. from *Crassostrea ariakensis*, *Bonamia* sp. from *Ostrea angasi* and *Bonamia* sp. from *Ostrea puelchana* seem marginal.

Taxonomy of Bonamia species

In biology “species” is one of the fundamental units for classification. Species must be morphologically and genetically defined to permit adequate diagnosis, epidemiological studies and impose controls on movements. As *B. ostreae* and *B. exitiosa* are notifiable to the EU and OIE presence of these organisms has implications for the shellfish trade.

In the modern concept of species are species separately evolving metapopulation lineages (de Queiroz 2005). The definition of species can be based on morphology/ultrastructure, host range, pathogenicity, geographical distribution, genetic diversity and/or a combination of these factors. Studies with bacterial species show that species defined by taxonomy in many cases correspond to well-resolved sequence clusters (Fraser et al. 2009). On the other hand the studies also show that there is no universal cut off (level of divergence) that characterizes a species. Therefore, additional characteristics (as described above) will have to be taken into account to make a justified decision on the level of divergence used as cut off. As this value is an artificial decision it can in due time be subjected to new advances in the concerning field of research.

B. ostreae and *B. exitiosa* species can be readily distinguished based on ultrastructure and genetic diversity. On the other hand it is not so clear whether *Bonamia exitiosa*-like microcells consists of different strains of one species or the group consists of multiple species (Lormann et al. 2009). The nearly identical ultrastructure and genetic sequences suggest strains of the same species. However the sequence data is limited to one gene and the microcells are described in different hosts and with a different geographical distribution.

Current knowledge on *B. exitiosa*-like microcells to be used for taxonomic identification is limited. For most of the species in this *B. exitiosa*-like group relative few data on histology, ultrastructure and molecular has been available for species and not sufficient to unequivocally discriminate between the species.

Although in different hosts a different histopathology has been described (e.g. *B. exitiosa* infection of *O. chilensis* and *O. angasi;* Hine: *unpub. obs.*), histopathology is not suitable tool for species identification. Moreover, the differences observed could be host mediated.

On the other hand ultrastructural observations have been made on the southern hemisphere "isolates", but not all of these have been published. As far as published is the ultrastructural morphology between the *B. exitiosa*-like microcells nearly identical and also here the observed differences can be host defined.

With regard to molecular identification more gene sequences will probably become available of different "isolates" in the future, however the current knowledge on genetic diversity within *B. exitiosa*-like microcells is based only on the ribosomal RNA gene complex.

Host species

The taxonomic status of the *Ostrea* species and its relation with *Ostreola* species is not settled and this hampers an assessment of the hazard of introduction and spread of new exotic strains of *Bonamia* to the EU.

As described by Heasman et al. (2004), the relationship between *O. angasi* and *O. edulis* remains unresolved. Possibly the two species are different lineages of the same species. Introduced in Australia from Europe the last 200 years.

The occurrence of a spore-forming species of *Bonamia, B. perspora*, in *Ostreola equestris* in North Carolina, U.S.A. has turned attention to this small oyster host. Lapègue et al. (2006) proposed a revision of the Ostreinae species. It consists of incorporation of the genus *Ostreola* in the genus *Ostrea* considering the very close relationship between *Ostreola equestris* and *Ostreola stentina* with *Ostrea stentina* based on 16S rDNA and cytochrome oxidase subunit I (COI) sequences. In addition to this, Shilts et al. (2007) typed samples from oyster population for 16S rDNA, COI and ITS-1 markers and phylogenetically analyzed the genotypes of flat oyster populations *O. puelchana, O. chilensis, O. equestris*, *O. stentina*, *Ostrea aupouria* and *Cryptostrea permollis*. The authors suggest *O. equestris* and *O. aupouria* to be synonymus to *O. stentina*.

Furthermore, examination of *O. aupouria* in New Zealand has produced a PCR product resembling *B. exitiosa/B. roughleyi* (Hine *pers. comm.*). In this light, it has been realised that the near indistinguishable oyster species *O. aupouria*, *O. equestris* and *O. stentina* occur in association with *B. exitiosa*-infected oysters in Australia, New Zealand, Argentina, Spain and the Mediterranean. To what extend these oysters may act as a global source of *B. exitiosa* needs to be established.

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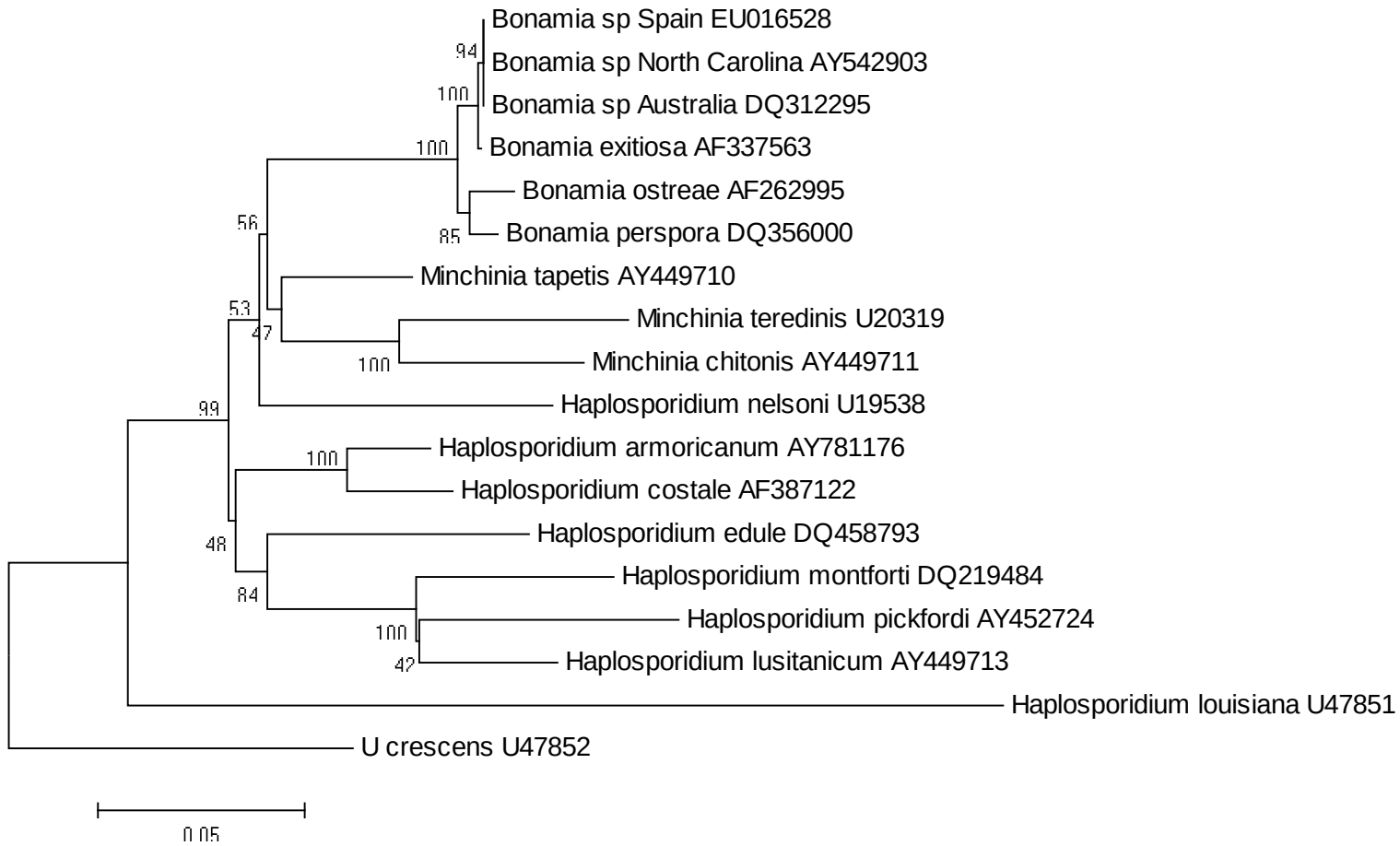

Figure 1. Neighbor-Joining phylogenetic tree of members of the family Haplosporidia based on 18S SSU rDNA sequences. GenBank numbers of used sequences indicated in parenthesis. Alignment of sequences using the program Clustal X, phylogenetic analysis carried out with Mega 4.0. Bootstrap consensus tree inferred from 10,000 replicates. Percentage of replicate trees in which the associated taxa cluster together are shown next to the branches. Evolutionary distances calculated with Jukes-Cantor method. Total of 1441 positions used in final dataset.

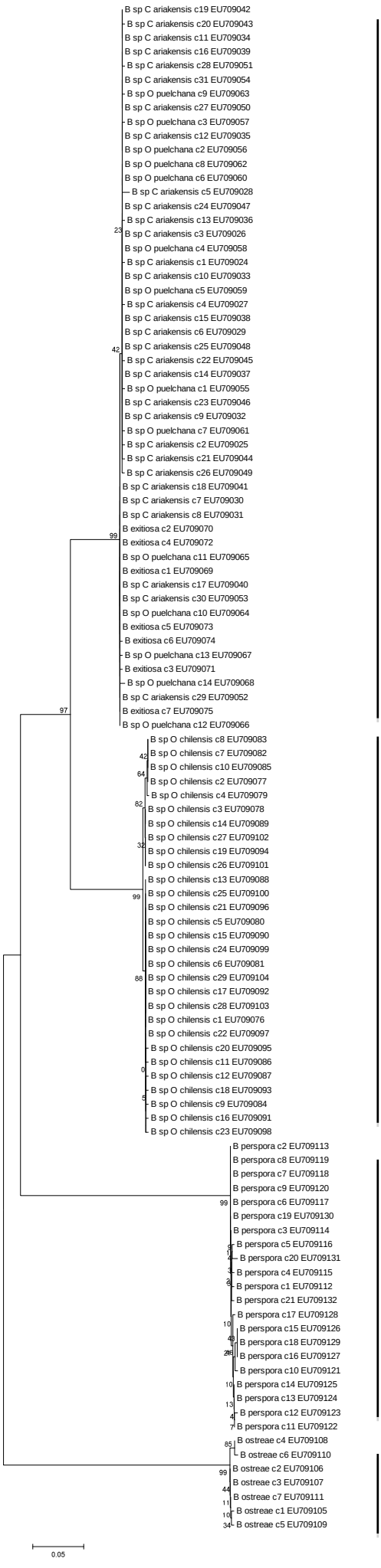

Figure 2. Neighbor-Joining tree based on available 497-550 bp fragment of the partial 18S rDNA, ITS1, 5.8S rDNA, ITS2 and partial large subunit rDNA sequence of *Bonamia* species. GenBank numbers of used sequences indicated. Alignment of sequences using the program Clustal X, phylogenetic analysis carried out with Mega 4.0. Optimal tree shown (unrooted). Percentage of replicate trees in which the associated taxa cluster together are shown next to the branches. Evolutionary distances calculated with Jukes-Cantor method. Total of 426 positions used in final dataset.

Bonamia exitiosa in Ostrea chilensis (New Zealand)
Bonamia sp. in *Ostrea angasi (Australia)*
Bonamia sp. in *Ostrea puelchana (Argentina)*
Bonamia sp. in *Crassostrea ariakensis (NC, USA)*

Bonamia sp. in *Ostrea chilensis (Chile)*

Bonamia perspora in *Ostreola equestris (USA)*

Bonamia ostreae in *Ostrea edulis (USA)*

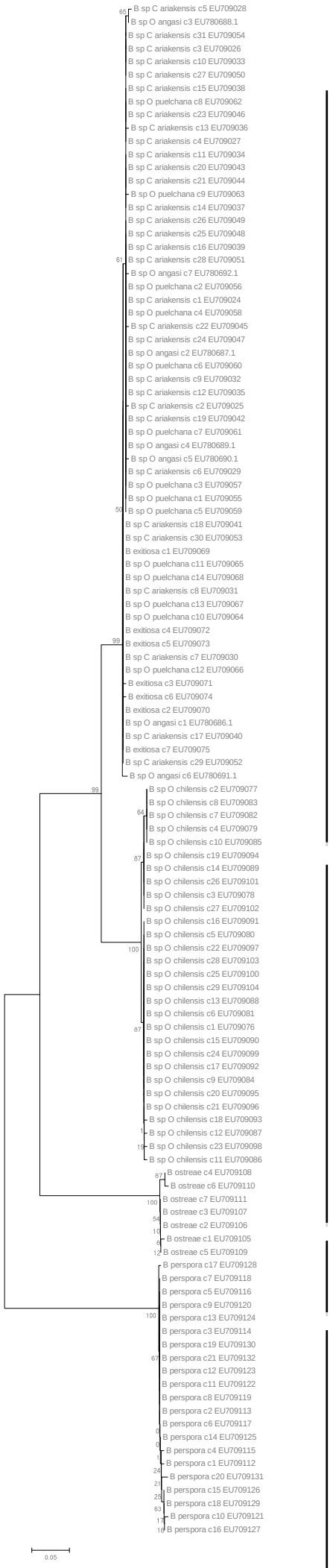

Figure 3. Neighbor-Joining tree based on available 345-550 bp fragment of the partial 18S rDNA, ITS1, 5.8S rDNA, ITS2 and partial large subunit rDNA sequence of *Bonamia* species. GenBank numbers of used sequences indicated. Alignment of sequences using the program Clustal X, phylogenetic analysis carried out with Mega 4.0. Optimal tree shown (unrooted). Percentage of replicate trees in which the associated taxa cluster together are shown next to the branches. Evolutionary distances calculated with Jukes-Cantor method. Total of 272 positions used in final dataset.

Bonamia exitiosa in *Ostrea chilensis (New Zealand)*
Bonamia sp. in *Ostrea angasi (Australia)*
Bonamia sp. in *Ostrea puelchana (Argentina)*
Bonamia sp. in *Crassostrea ariakensis (NC, USA)*

Bonamia sp. in *Ostrea chilensis (Chile)*

Bonamia ostreae in *Ostrea edulis (USA)*

Bonamia perspora in *Ostreola equestris (USA)*

6.6. Annex F

Database/GIS workshop programme & participants

Programme

Day 1 · Wednesday 21 October 2009

Timing	Activity	Person in charge
9.00 - 9.15	Official opening	V. Caporale, Director IZSA&M
9.15 - 9.30	Introduction and welcome	R. Lelli, Deputy Director IZS A&M
9.30 – 9.45	Presentation of IZS A&M	P. Calistri, IZS A&M
9.45 - 10.00	Project introduction	F. Berthe, EFSA Coordinator
10.00 - 10.20	Project presentation	B. Hill, Project Coordinator
10.20 - 10.40	Scientific review methodology, epidemiological database content	A. Reese, CEFAS
10.40 – 11.00	*Coffee break*	-
11.00 – 11.30	Comments on conducting the review process, with Bonamia as an example	M. Engselma, Central Veterinary Institute of Wageningen UR
11.30 – 13.00	Scientific review discussion	All participants
13.00 – 14.00	*Lunch*	-
14.00 – 15.30	Presentation of database structure and web-GIS application. Goals, Objectives, Scheduled activities of the Training programme	C. Ippoliti, P. Calistri, IZS A&M
15.30 – 16.00	*Coffee break*	-
16.00 – 17.00	Database and web-GIS training	C. Ippoliti, IZS A&M

Day 2 · Thursday 22 October 2009

Timing	Activity	Person in charge
9.00 - 10.45	Discussion and Conclusions	All participants
10.45 - 11.00	Future Tasks, meetings and deadlines	EFSA Coordinator
11.00 - 11.30	*Coffee break*	-
11.30	*Departure*	-

Invited participants

Name	Organization	Location
Barry Hill	CEFAS	Weymouth, UK
Allan Reese	CEFAS	Weymouth, UK
Marc Engselma	Central Veterinary Institute of Wageningen UR	Lelystad, NL
Olga Haenen	Central Veterinary Institute of Wageningen UR	Lelystad, NL
Grant Stentiford	CRL for crustacean diseases	Weymouth, UK
Helle Frank Skall	CRL for fish diseases	Aarhus, Denmark
Isabelle Arzul	CRL for mollusc diseases	La Tremblade, France
Franck Berthe	EFSA	Parma, IT
Ana Afonso	EFSA	Parma, IT
Francesca Riolo	EFSA	Parma, IT
Jane Richardson	EFSA	Parma, IT
Paolo Calistri	IZS A&M	Teramo, IT
Carla Ippoliti	IZS A&M	Teramo, IT
Alessio Di Lorenzo	IZS A & M	Teramo, IT
Lara Savini	IZS A & M	Teramo, IT

6.7. Annex G

Characteristics of the database and web-GIS application

Database structure

The database is constituted by a main table, where the epidemiological data are stored, and some supporting tables where values for standardized variables are included. Another table containing epidemiological data reported for specific locations is also available.

The diseases are coded in a table, and this allows addition in the same database of information on other diseases, if required by future EFSA activities. The same philosophy is applied to the other supporting tables, where new codes can be added without changes in the whole database; descriptions can also be modified when required.

The main table, named DISEASES_PRESENCE, stores all the occurrences of the diseases, recording them by geographical areas (Provinces, Regions, Countries): the most detailed geographical level available in the literature has been entered by the experts. This table allows the depiction of the worldwide epidemiological situation of the VHS and *Bonamia* spp.

The second epidemiological table, named DISEASES_OUTBREAKS, includes data on occurrence referred to point locations (for which coordinates are known and correct). This table collects additional data, i.e. it reports all the details of point locations, recorded in the papers reviewed.

Then, for each polygon in DISEASES_PRESENCE, *n* points can be present in DISEASES_OUTBREAKS, but this is not mandatory; and for any point location in DISEASES_OUTBREAKS, the corresponding geographical area is reported as infected in DISEASES_PRESENCE.

Figure 1 shows the structure of the database for the collection and storage of information on disease occurrences.

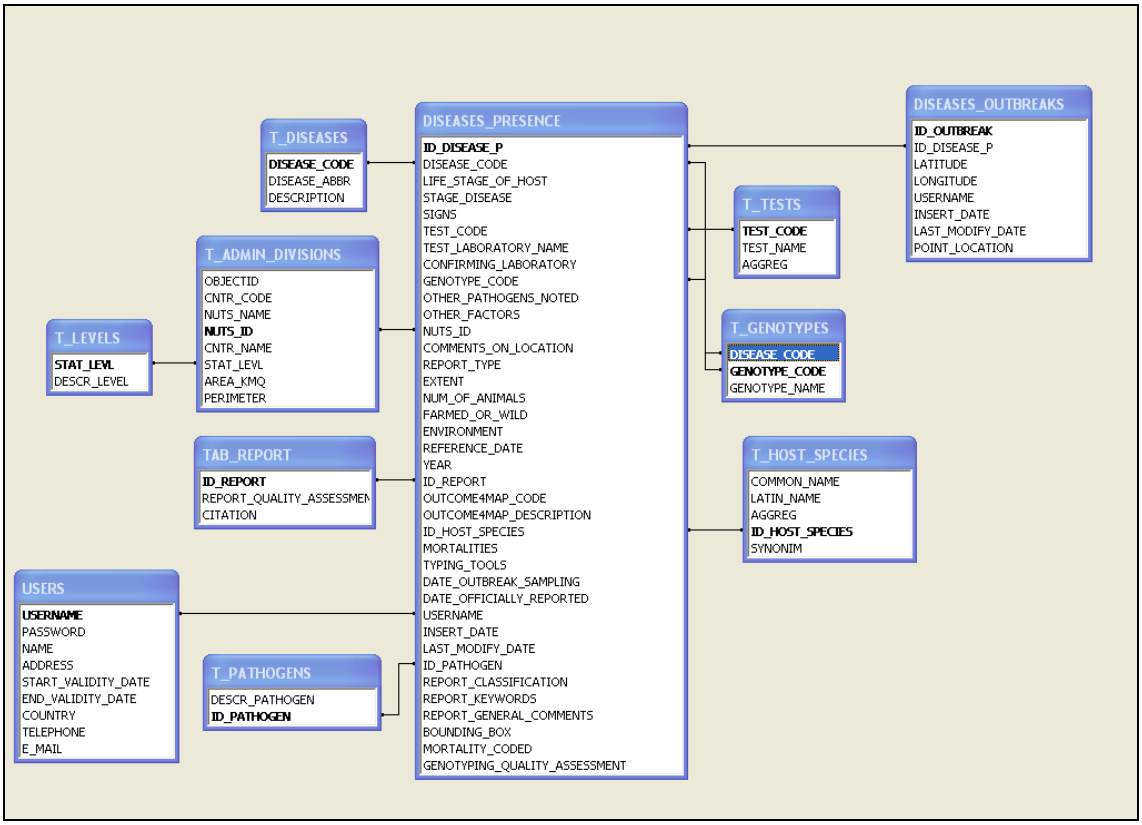

Figure 1: Database structure

The supporting tables contain codes and names of specific variables (host, pathogens, region names, etc) and the related values are described in the following tables (Tables 1-8). The values in the tables are a sub-sample of the values coming from the scientific review of the diseases carried out by the reviewers, unless indicated as example data.

Table 1: T_DISEASES - Codes and names of the diseases

DISEASE_CODE	DISEASE_ABBR	DESCRIPTION
32	VHS	VIRAL HAEMORRHAGIC SEPTICAEMIA
52	IBE	INFECTION WITH BONAMIA EXITIOSA
53	IBO	INFECTION WITH BONAMIA OSTREAE

Table 2: T_HOST_SPECIES - Codes and names of affected species (host), both in common and Latin name. Sub sample of original data

This table contains the values derived from the literature review carried out by the experts AND the species reported in the Scientific Opinion of the AHAW Panel on a request from the European Commission on aquatic animal species susceptible to diseases listed in the Council Directive 2006/88/EC- The EFSA Journal (2008) 808, 1-144.

In the Latin name column, the more recent names of the species have been considered.

ID_HOST_SPECIES	COMMON_NAME	LATIN_NAME	SYNONIM
152		Cerastoderma edule	Cerastoderma edule, Cardium edule
26	Rainbow trout	Oncorhynchus mykiss	Rainbow trout (Oncorhynchus mykiss), Rainbow trout. Oncorhynchus mykiss (Walbaum).

Table 3: T_PATHOGENS - Codes and names of pathogens

ID_PATHOGEN	DESCR_PATHOGEN
1	Bonamia exitiosa
2	Bonamia ostreae
3	Bonamia sp.
4	VHSV

Table 4: T_GENOTYPES - Genotype codes and genotype names for VHSV

DISEASE_CODE	GENOTYPE_CODE	GENOTYPE_NAME
32	I	Genotype I
32	II	Genotype II
32	III	Genotype III
32	IV	Genotype IV
32	99	Not specified

Table 5: T_TESTS - Laboratory tests for diagnosis of disease. Sub sample of original data

TEST_CODE	TEST_NAME
1	Cytocentrifugation
2	Electron microscopy
3	ELISA
4	Heart smear
5	Histopathology

Table 6: T_ADMIN_DIVISIONS - Geographical administrative subdivisions, sub-sample data. This table contains all the geographical units of the World to be displayed as infected in the web-GIS. The key field is NUTS_ID, acting as link between epidemiological data and geographical features.

The table contains five geographical levels (reported in the table T_LEVELS):

1. STAT_LEVL_=0 corresponds to the Country level (for all the World)
2. STAT_LEVL_=1 are macro-areas within the Country (available for EU Countries only)
3. STAT_LEVL_=2 corresponds to the Regions level (for all the World)
4. STAT_LEVL_=3 corresponds to the Provinces level (available for EU Countries only)
5. STAT_LEVL_=4 corresponds to the ICES level (available for Northern Atlantic and North Sea only)

STAT_LEVL	CNTR_CODE	CNTR_NAME	NUTS_ID	NUTS_NAME
0	IT	Italy	IT	Italia
1	IT	Italy	ITD	Nord-Est
2	IT	Italy	ITD5	Emilia-Romagna
3	IT	Italy	ITD52	Parma
4	H2	Western Mediterranean Sea	01E5	ICES 01E5

Table 7: TAB_REPORT - Information on the publications (quality assessment indicated by the reviewers). Example data

ID_REPORT	REPORT CITATION	REPORT_QUALITY_ASSESSMENT
1	WAHID-Interface (2007) Infection with Bonamia exitiosa, Spain 17/10/2007, OIE	Definite
2	Aaaa (2008). First detection of the protozoan parasite Bonamia exitiosa (Haplosporidia) infecting flat oyster Ostrea edulis grown in European waters. Aquaculture xx, pp	Probable
3	Bbbbb. (yyyy). Epizootiological aspects of VHS in Spain. In: Fish Diseases - Enfermedades de los Peces - Fourth COPRAQ Session. (ACUIGRUP, Ed.), pp. ATP, Madrid.	Possible
4	Ccc. and Ddddd (1985). Occurrence of VHS virus in wild white fish (Coregonus sp.). Zbl. Vet. Med. vol pp.	Doubtful

Table 8: TAB_USERS – Users enabled to insert or update database table content. Example data

USERNAME	PASSWORD	NAME	ADDRESS	START VALIDITY DATE	END VALIDITY DATE	COUNTRY	TELEPHONE	E_MAIL
ENGELSMA	*****	Marc	WUR	01-Sep-09	31-Dec-30	The Nethrlands	+31 (3) 20 2380000	
REESE	xxxx	Allan	Cefas	01-Sep-09	31-Dec-30	United Kingdom	+44 (0)1305 206000	

Geographical data

The occurrences of VHS and *Bonamia* spp. infections are shown on a map, and the related epidemiological data is available through queries.

The diseases occurrence is represented as:

- polygon-based layers based on administrative boundaries at Country/region/province level, and

- point-based layers (specific locations) geo-referenced through latitude and longitude data, when geographical coordinates of the occurrence point location are available.

For the latter geographical data type, the layer is based directly on the alphanumerical values of latitude and longitude in the DISEASE_OUTBREAKS table.

For the administrative geographical units of reference (polygon based layer), a unique geographical layer with World extent has been created. The available levels of representation for the disease occurrence and the related source follows:

- for EU countries, the Nomenclature of Territorial Units for Statistics – NUTS - Level 0, 1, 2, 3, as indicated in the Regulation (EC) No. 1059/2003 of the European Parliament and of the Council of 26 May 2003 and following amendments (figure 2) has been downloaded from

European Commission website (http://ec.europa.eu/eurostat/ramon/nuts/home_regions_en.html);

- for the rest of the World, the ESRI shapefile Level 2 has been used, available in ESRI CD DATA&MAPS;

- for North Europe and North Atlantic Seas, International Council for the Exploration of the Sea (ICES) polygons have been used. This is a grid covering the sea of North Atlantic Ocean, the Baltic Sea, most of the Mediterranean and the Black Sea (figure 3). It is freely available at http://www.ices.dk/aboutus/icesareas.asp. It was considered as level 4 in the geographical database.

The Geographic Coordinate System is WGS_1984 lat/long and datum WGS_1984 and the geographical extension of the map is: World.

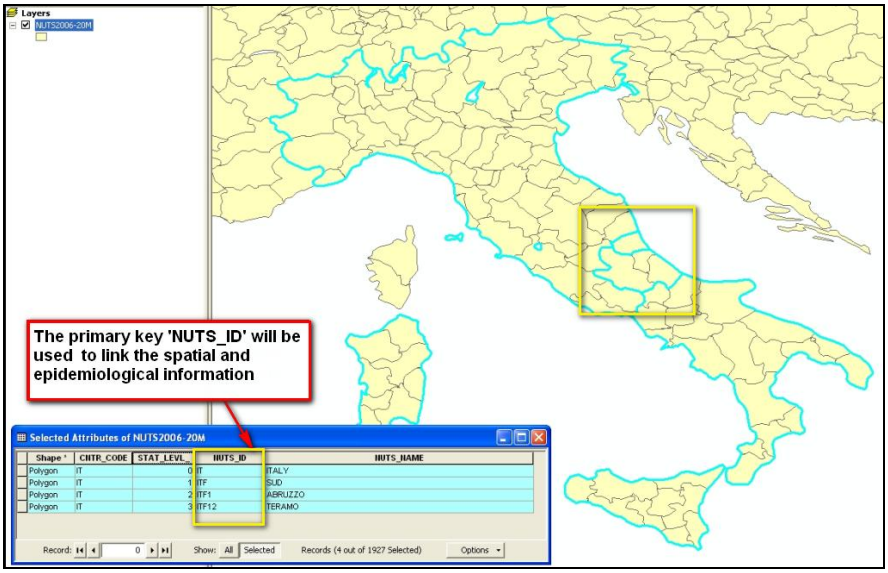

Figure 2: Example of the NUTS data: Level 0, 1, 2, 3

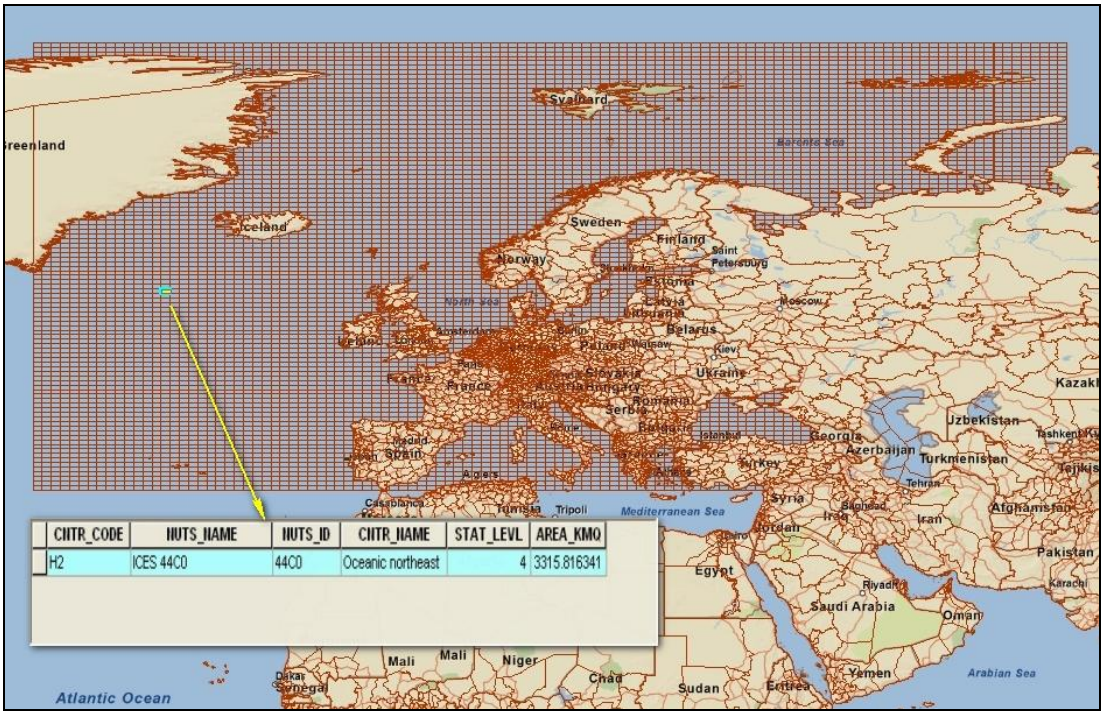

Figure 3: Geographical extent and attributes of ICES data

Link between alphanumeric and geographical data

The alphanumeric data collected (in DISEASE_PRESENCE) has been linked with the geographical layer of the administrative units (SDE layer), to be shown on the web-GIS. The link is based on the NUTS_ID field, present in both tables.

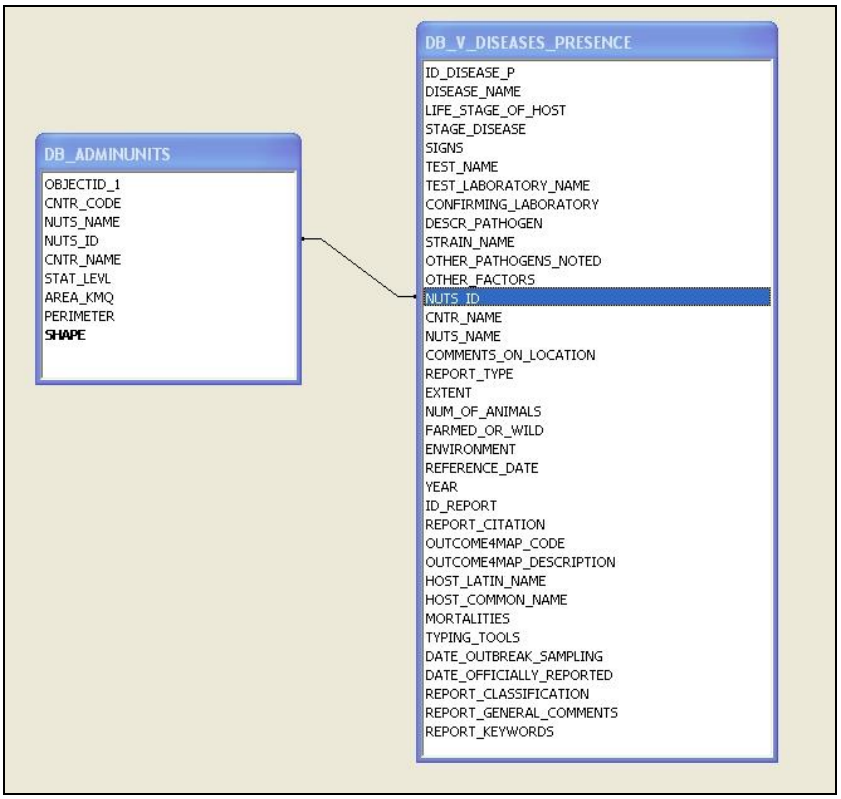

Figure 4: Link between geographical data (ADMINUNITS) and epidemiological information (DISEASES_PRESENCE)

The epidemiological data collected

The MS Excel files provided by the reviewers have been processed and transferred into the geo-database (i.e. the database completed by the geographical levels).

To this objective, for each field in the data entry spreadsheet, a new column was created, and functions where developed to standardize the values reported.

The ideal process at this point would have been to:

- correct any apparent typographic errors and check with the reviewer whether these were in the original source and should therefore be retained in sheet 1;

- review all terms used in original sources and agree the standard terms to be retained or substituted in the supporting tables;

- compare across reviewers to ensure consistency.

For Infection with *Bonamia* species, a basic/preliminary review consistency process was performed by Marc Engselma and Carla Ippoliti through e-mail exchange and a meeting in mid September 2009.

All data on Bonamia and VHSV diseases have been uploaded into the database: the values that needed an interpretation by experts have been indicated as “999” code (as for not found, not indicated, not reported values) and the year of occurrence not clearly evident was indicated as 1900.

The Web-GIS application

The application has been designed to visualise the data stored in the database and customise (user-defined) maps, through GIS tools. The information accessible are: viral haemorrhagic septicaemia (VHS) and *Bonamia* spp. infections.

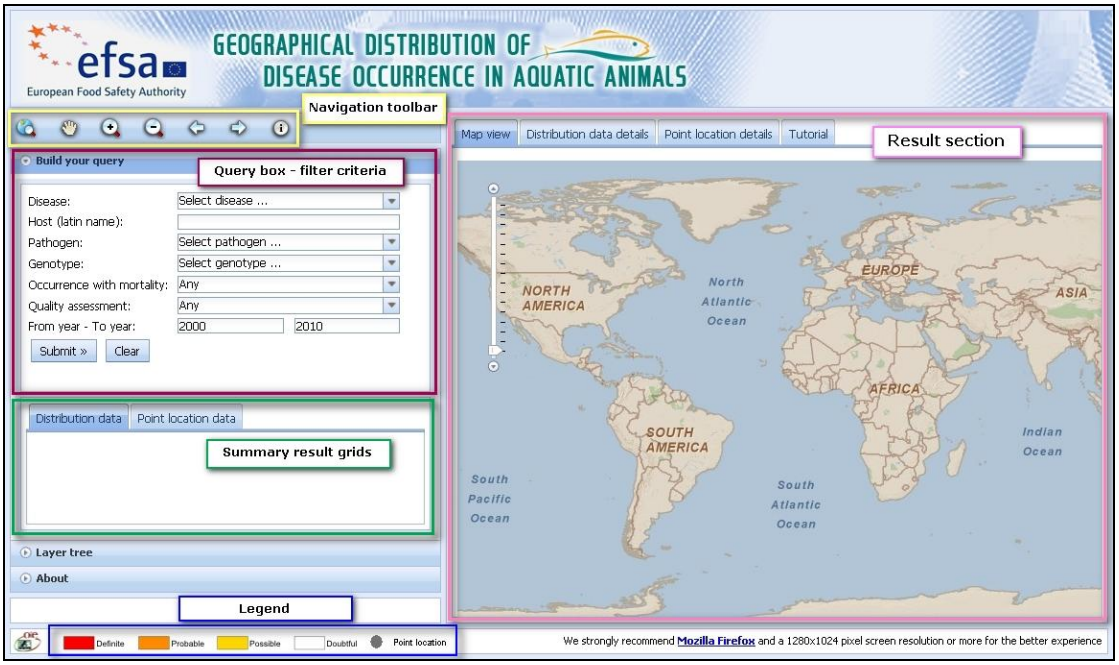

Figure 5: Interface of the demo Web-GIS application developed

The web-GIS interface application (figure 5) is composed by:

- a navigation toolbar with standard geographical tools: zoom in, zoom out, zoom to full extent, zoom to previous view and pan to navigate in the map;

- an identify tool to retrieve epidemiological and geographical information on a location by clicking on the map (see details below and figure 8);

- a query builder box: this is the first fundamental tool when interacting with the system. The user access this part to create his own set of data (personalization of the map), filtering data according to multiple criteria: disease, pathogen, genotype, time period, etc;

- a map results view: shows the results of the query performed as a thematic map with polygons and points;

- a table results view for distribution data: in the tab "distribution data details", over the map, the grid contains the complete information on disease occurrence, available into the database and responding to the chosen criteria,

- a table result view for point location data: in the tab "point location data details", over the map, the grid contains the complete information on point location data, available into the database and responding to the chosen criteria,

- query result summary for disease occurrence: in the tab "distribution data", under the query box, the grid contains a summary of the information on disease occurrence available into the database responding to the chosen criteria;

- query result summary for point location occurrence: in the tab " point location data", under the query box, the grid contains a summary of the information available into the database on point location data responding to the chosen criteria;

- a tutorial document;

- a “layer tree” section to be used for the personalisation of the visible layers on the map. The possible interactions are:

    - layer list: to check/uncheck each layer in the list, to display/not it on the map. The list contains: disease outbreaks (point location data), disease presence (polygon location of the disease), administrative divisions (the base layer with all the geographical units), satellite and shaded relief (background images);

    - select: in the dropdown list to choose a layer, then to click on ‘zoom to layer’ button to enlarge the map to its extension;

    - change layer transparency: in the ‘select’ dropdown list to choose a layer, then to click on the slider to modify the layer transparency;

- a legend explaining the colors of the polygons: red stays for definite, orange probable, yellow possible, transparent doubtful. The point layer has the same legend;

- coordinates in lat/long decimal degrees of the current position of the mouse;

- recommended software for the best performance of the web-GIS application (the web browsers supported are Internet Explorer 7.0 or greater, Chrome, Mozilla Firefox – recommended -).

Tutorial

How to query

In the section "Build your query", the user creates his own set of data to be shown on the map (personalization of the map) and to be viewed in the table grids, filtering data according to multiple criteria.

The filters retrieve the values directly from the epidemiological database, and some of them are linked together, i.e. pathogens values appearing in the list depend on the chosen disease, the genotype values depend on the chose pathogen (available for VHSV only). For this reason, it is important to choose the criteria in the order they appear.

The mandatory filters to be set are indicated by (*):

1. disease (*): to choose the disease of interest among the ones reported into the database (the list is automatically populated with the values into the database);

2. host (Latin name): to type the Latin name, or part of it, of an affected species; if not filled in, all the values in the database are considered. In this filter, and in the table on which it is based on, the more recent names of the species have been considered;

3. pathogen (*): to choose the pathogen of interest among the ones reported into the database for the chosen disease;

4. genotype (*): to choose the genotype of interest among the ones reported into the database only for the Viral Haemorrhagic Septicaemia Virus pathogen;

5. occurrence with mortality: to choose if the user wants to check for occurrence with mortality reported or not, or to leave “any” to see all the records;

6. quality assessment of the paper: to choose the quality assessment indicated for the report among the ones reported into the database;

from year - to year (*): to choose the time interval of interest, typing the year start the period and the year ending it, in format yyyy.

Then submit the query to get the results.

How to get the query results

After submitting a query to the system, the user can retrieve the results in many ways:

a) on the map: the "map view" tab, in the right part of the page, shows the results as thematic map with polygons and points;

b) in the "distribution data details" tab and "point location data details" tab (tabs over the map): the grids contain the complete information available into the database responding to the chosen criteria;

c) in the "distribution data" tab and "point location data" tab (tabs under the query box): the grids contain a summary of the information available into the database responding to the chosen criteria.

Click on the names of the fields to order (ascending/descending) the contained values. Drag and drop the names of the fields in the grids to change the order of the columns.

How to export the query results

The user can export the data details result of the query to a local computer in table format. This tool is available for distribution data and point location data.

At the bottom of "distribution data details" tab and "point location details" tab, click on "CSV formatted response" (Figure 7) and then follow this steps:

I. right click into the page and chose the - select all - option,

II. copy the selection,

III. paste the content of the page in a blank document and save it as *.txt file,

IV. open it through MS Excel, choosing pipe as field separator.

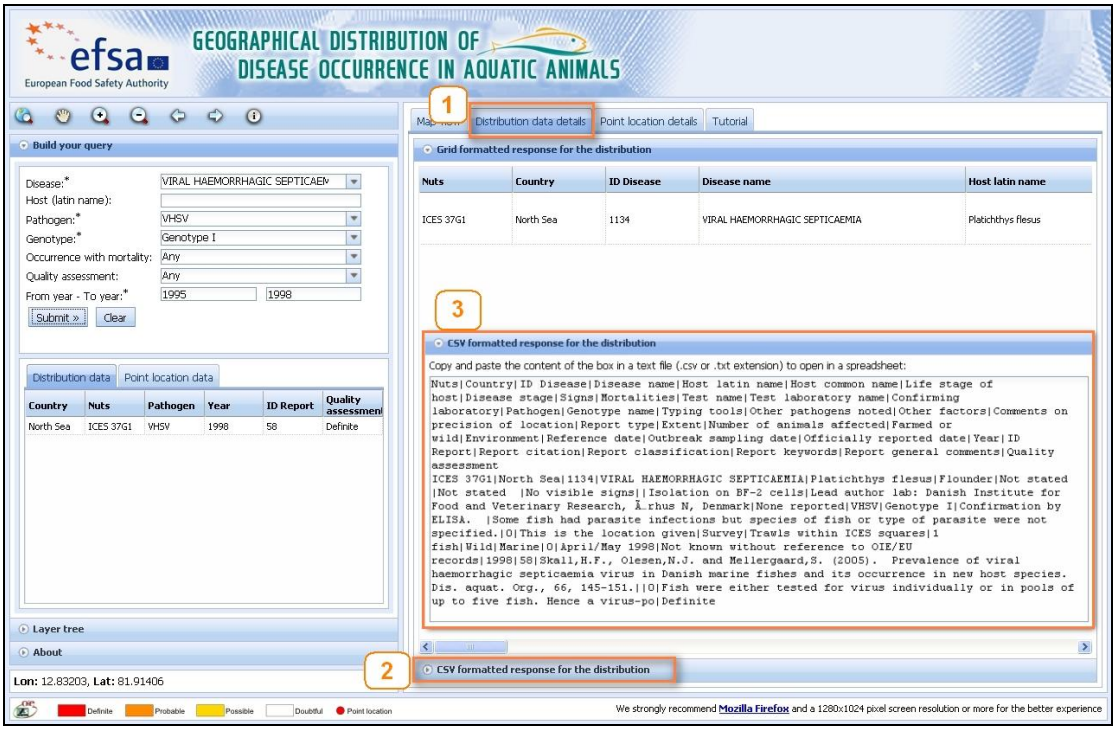

Figure 7: After performing a query, go to "distribution data details" tab or "point location details" tab (1), click on "CSV formatted response" at the bottom of the page (2), and a new panel will open (3). Follow the steps I-IV above mentioned to download the csv formatted results.

How to use the identify tool

The identify tool on the navigation toolbar can be used to retrieve information for a specific element on the map, by clicking on it. The following figure shows an example.

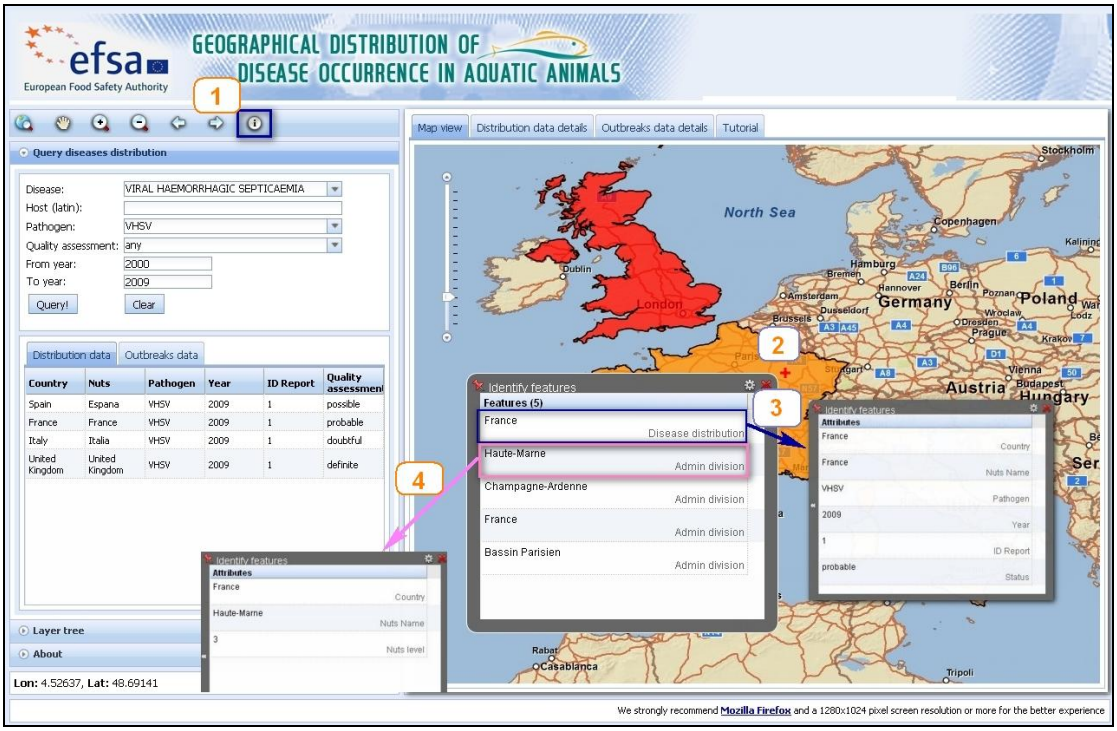

Figure 8: Example data. Click on the identify tool (1) to retrieve all the information on the location clicked (2), either disease distribution summary (3, in blue) or basic administrative information (4, in pink).

Technical specifications

The Web-GIS application is composed by:

- a server-side: ArcGIS Server 9.3 Standard Enterprise for the Java Platform;

- a client-side: ArcGIS Server 9.3 Javascript APIs (v. 1.4 from the online ESRI serverapis).

The client is platform independent and is composed by html and javascript code (includes Dojo toolkit objects and syntax. Dojo is the base on which ArcGIS 9.3 Server Javascript APIs are built on).

The client application is visible from remote browsers when exposed by a Web-Server (e.g. IIS, Apache, etc.).

The web-GIS is based on an ArcMap project, containing the data shown in figure 9.

Disease outbreaks is an event-theme layer, i.e. it is a geographical layer build on the fly by the software reading the latitude and longitude fields (in the view v_diseases_outbreaks).

Disease distribution layer is the result of the join between administrative units (geographical file) and v_disease_presence (alphanumerical table) containing the epidemiological information.

These layers are subject to the filters on the web-GIS application.

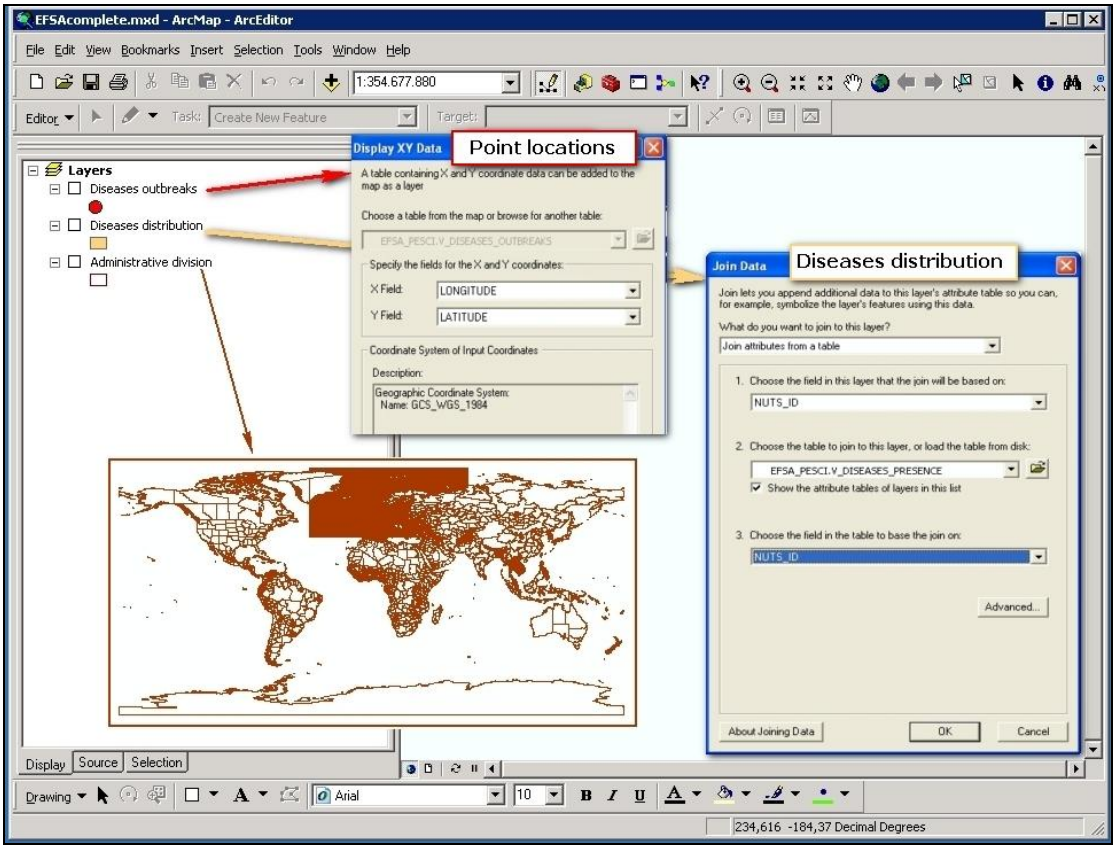

Figure 9: The ArcMap project, base of the web-GIS application

All the layers should be turned off when saving the .mxd project. The web client application will manage the turn on and visualization of each single layer.

The ArcMap .mxd project is then published as map service through ArcGIS Server Manager (figure 10).

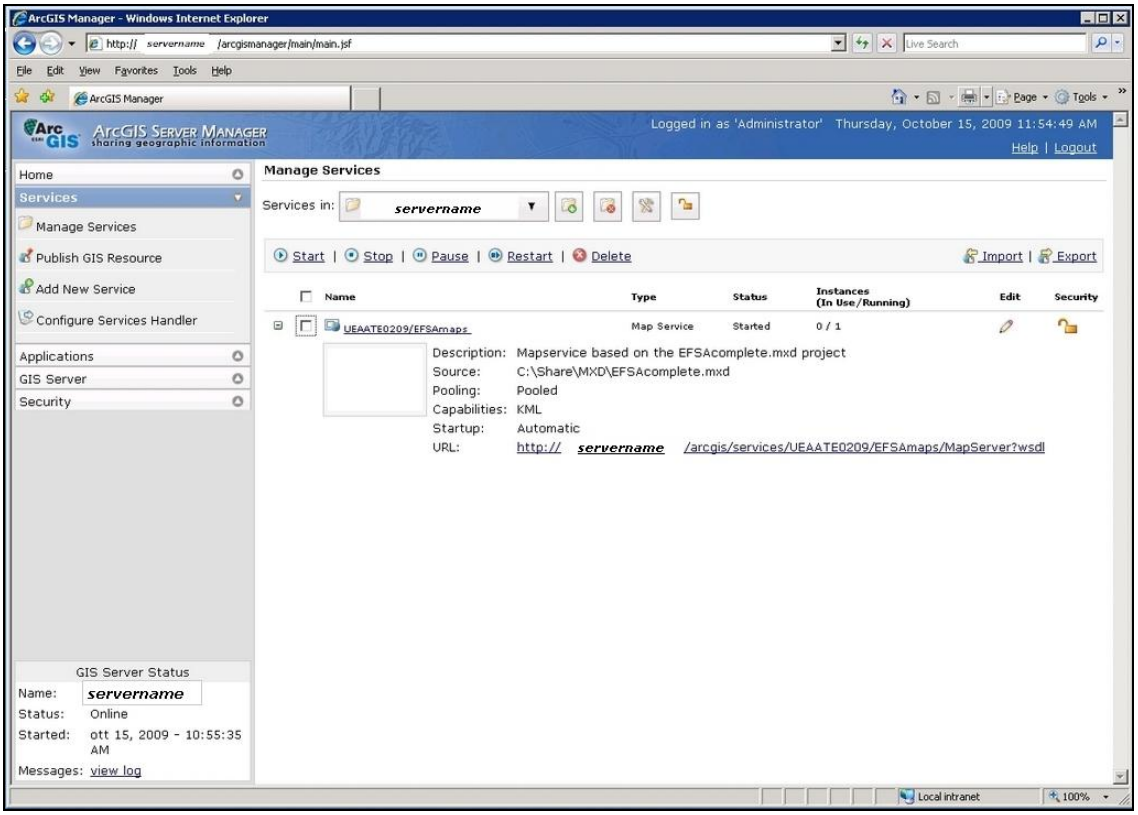

Figure 10: Summary of the service in ArcGIS Server Manager

Image background layer are available directly on-line, at ESRI ArcGIS Server service on services.arcgisonline.com.

The map service, the background images, and all other components are published together in an html page.

At this stage, the web-GIS is available at the web address http://yourarcgisserverurl/arcgis/rest/services. The address should be available through any internet browser and should appear as in the following image.

Figure 11: REST ArcGIS Server url page

Procedure and documents

The overall scheme is reported in figure 12: epidemiological data are hosted in a relational database (Oracle 10g) and the geographical data are managed by ESRI ArcSDE 9.3. ESRI ArcGIS Server 9.3 is the software to publish the map service on the World Wide Web.

The releases of Oracle and ESRI meet all the project and technical requirements and they are available at EFSA.

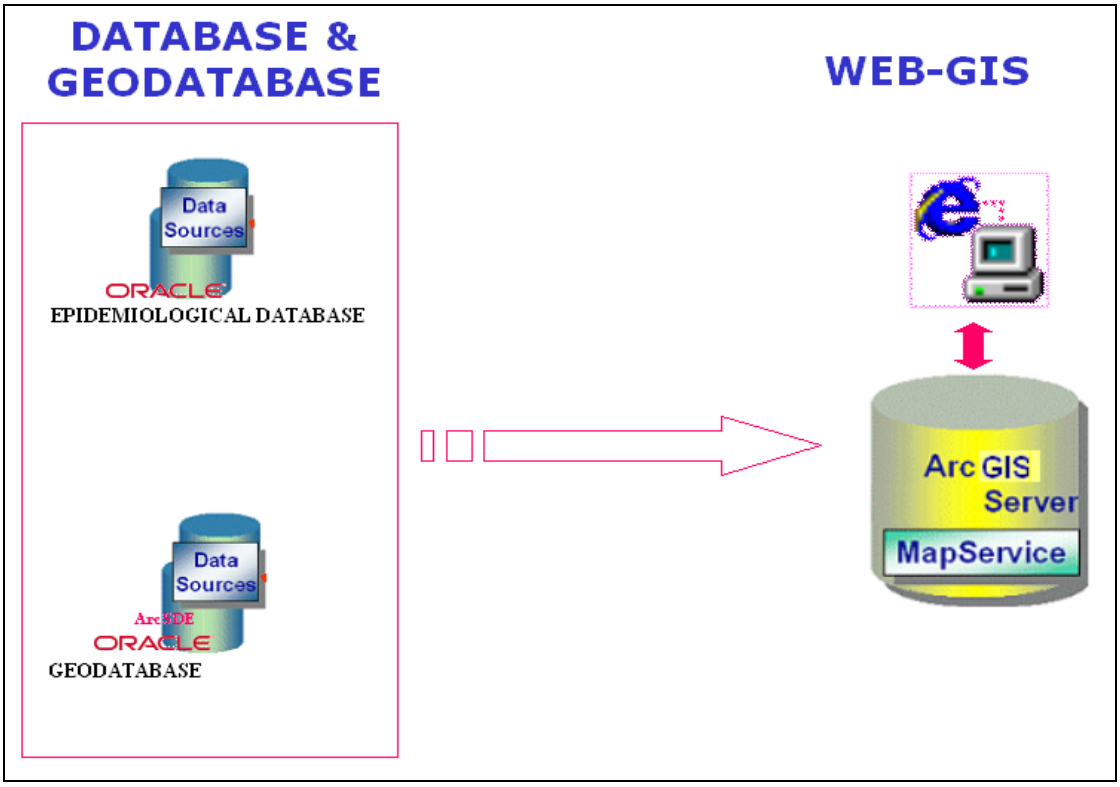

Figure 12: Overall scheme of database and web-GIS structure

Instructions to install the application

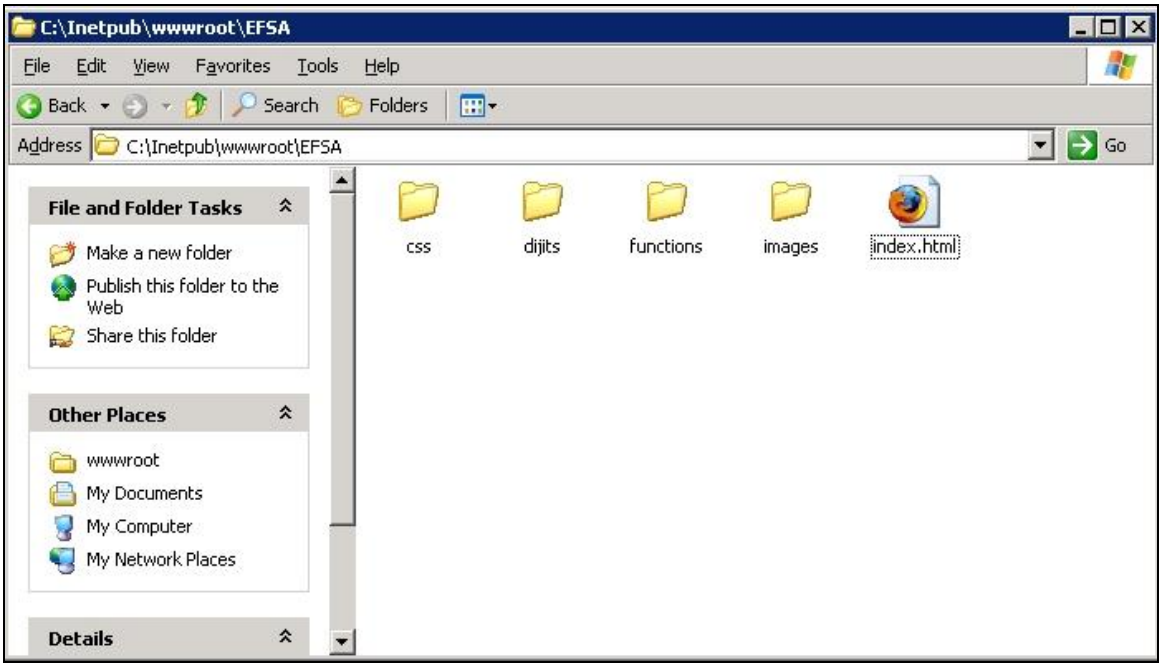

Figure 13: client application directory content

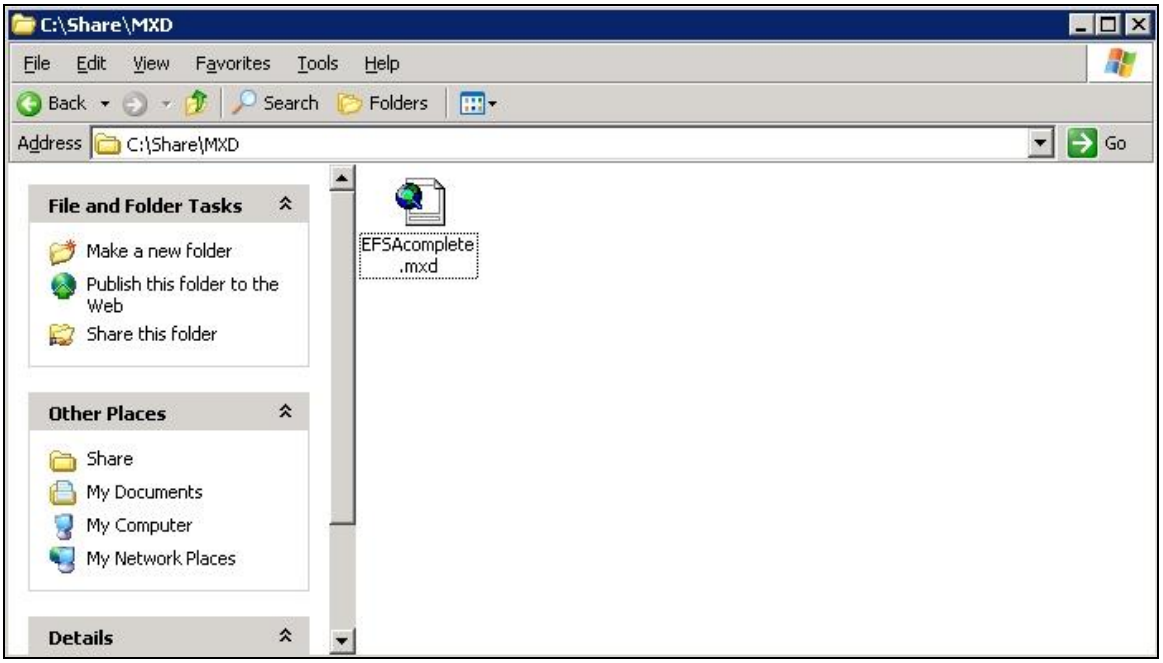

Figure 14: ESRI ArcGis Arcmap project

REQUIRED SOFTWARE COMPONENTS
==========

1) Oracle 10g
2) ArcSDE 9.3
3) ArcGIS Desktop 9.3
4) ArcGIS Server 9.3
5) IIS WebServer (included in Microsoft Windows)

STEP 1 - Restoring the Oracle dump
==========

Restore the Oracle schema export (exp_EFSA_data.dmp) coming from IZSA&M
It contains all the tables and the data (spatial and not-spatial) that the web-gis application needs to work on.

Create a spatial database connection to the new Oracle schema (in our demo the schema is efsa_pesci).

A copy of the administrative divisions geographical layer is also in a Microsoft Access personal geodatabase (EFSA_ADMINUNITS.mdb). In case of Oracle import error on layer, import it into your SDE database.

STEP 2 - MXD project
==========

Open the MXD Project with ArcMap coming from IZSA&M "EFSAcomplete.mxd " and verify that all layers from the Oracle/SDE database are visible. If not, set the data source as follows:

Diseases outbreaks: (layer 0) a non spatial view (V_DISEASES_OUTBREAKS) with lat/lon field used to generate the point location data as event theme.

Diseases distribution (layer 1) generated from the join (only matching records) between V_DISEASES_PRESENCE view and the ADMIN_DIVISION sde layer.

Administrative division (layer 2)
The ADMIN_DIVISION sde layer.

Turn off all the layers and save the project as EFSAcomplete.mxd

In case the MXD Project is not available create one as detailed above (see the figure 10).

STEP 3 - ArcGIS Server configuration
==========

Create an ArcGIS Server mapservice called EFSAmaps, using the EFSAcomplete.mxd as source.

Ask for the url: http://yourserver:8399/arcgis/rest and check if you see the service rest root with your EFSAmaps mapservice working.

STEP 4 – Web-GIS client installation
==========

Copy the EFSA directory provided by IZSA&M into your webserver document root.
Inside the index.html file there is a javascript function called init(), in which the content of the serviceURL global variable has to be substituted.
For example, the following is the content of the variable in our demo:
var serviceURL = http://picassoserver/arcgis/rest/services/EFSAmaps/Mapserver/";
Substitute picassoserver with the name of your server.

STEP 5 - Test the application at work
==========

Point your browser (Mozilla Firefox is the recommended one) to
http://yourserver/EFSA/index.html

UPDATING DATA – Future activities to maintain the system
==========

For future updates of disease epidemiological information, the only foreseen action is to populate the alphanumeric Oracle tables. It can be done without using GIS software.

GIS software is required only if the administrative divisions geometry needs to be modified (i.e. add or delete polygon features).

7. Glossary