Herpesvirus anguillae (HVA) isolations from disease outbreaks in cultured European eel, *Anguilla anguilla* in The Netherlands since 1996


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**Abstract**

Since 1996, Herpesvirus anguillae (HVA) infections were diagnosed 19 times at 14 Dutch eel farms with European eels (*Anguilla anguilla*). The eels showed various clinical symptoms, but predominantly apathy, haemorrhages in their skin and fins, ulcerative skin and fin lesions, haemorrhages in the gills, congested gill epithelium, a pale liver, and a mortality up to 10%. Eel gills were in more cases HVA positive than the corresponding organs. Therefore, gills should be sampled for HVA-isolations in diseased eels. Often a bad water quality induced the virus propagation. Eleven outbreaks occurred at water temperatures higher than 23°C; six outbreaks at water temperatures lower than 23°C. Decreasing the temperature to below 22°C worked well in 4 cases. The agent was isolated on Eel Kidney (EK-1) cells, and characterized as a herpesvirus with electron microscopy, typed by digestion of virus DNA with restriction enzyme HindIII, and its DNA fragments were analysed by gel electrophoresis. All Dutch HVA isolates showed a similar DNA pattern. Comparison of the DNA of the Dutch viruses and Japanese isolates revealed a small difference after digestion with HindIII, however.

**Introduction**

Herpesviruses have been isolated from different fish species (Hetrick and Hedrick, 1993). They have been isolated mostly from various skin infections in fish (Kelly et al., 1983a, 1983b; Ahne 1985; Hedrick et al. 1991; Anders & Yoshimizu 1994; Kobayashi & Miyazaki 1997), but also from systemic infections, like in case of Channel Catfish Virus (Fijan 1968). Lately, herpesvirus seem more and more related with gill infections (Bretzinger et al., 1999; Lee et al., 1999; Neukirch et al., 1999; Body et al., 2000; Hedrick et al., 2000).

In Asia, *Herpesvirus anguillae* (HVA) was isolated in 1985 from diseased Japanese eel *Anguilla japonica* and European eel *A. anguilla* (Sano et al. 1990), showing skin and gill erythema and necrosis of skin, gills and liver, with mortality rates of 1% and 6.8% respectively. Kobayashi & Miyazaki (1997) isolated HVA from Japanese eels with skin lesions, and tested the pathogenicity of HVA from cutaneous lesions of Japanese eel *A. japonica*. From 1988-1990, Ueno et al. (1992) isolated EHVF (Eel Herpesvirus in Formosa) from diseased Japanese eels in Taiwan, showing varicella on their skin surface. Ueno et al. (1996) proved, that HVA and EHVF were tightly clustered, based on syncytia formation, viral yields, cross reactivity in neutralization tests, and electrophoretic patterns of structural proteins. Lee et al. (1999) described gill filament necrosis in farmed Japanese eels infected with HVA.
In Europe, in 1985, Békési et al. (1986) demonstrated herpesvirus-like particles in skin lesions of European eel in Hungary, but the virus could not be isolated. In the period 1977-1992 herpesvirus was isolated from non diseased European eels in France (Jørgensen et al. 1994). Davidse et al. (1999) described the first isolation of HVA from systemically diseased European eels in The Netherlands. Organ suspensions which had been stored since 1996, from disease outbreaks, were found HVA positive. The typical herpesvirus morphology was found by electron microscopy. Van Nieuwstadt et al. (2001) proved, that HVA-replications in latently infected, seropositive eels are induced by stress. This paper describes 19 disease outbreaks due to a herpesvirus infection in farmed European eel *A.anguilla* since 1996 in The Netherlands.

**Materials and Methods**

**Necropsy**

Nineteen portions of live, diseased or non-feeding European eels, *Anguilla anguilla* from 14 eel farms were brought to our laboratory by the fish farmer for general diagnosis. The anamnesis of each disease case was recorded, including possible stress factors, like a bad water quality at the eel farm during the outbreak. The eels were checked for clinical symptoms, anaesthetized with metomidate (20 mg/l), and decapitated. Fresh smears of skin slime, and a piece of gill tissue were examined directly for parasites, fungi, and presence and numbers of bacteria, with a light microscope at 20-400 magnification. The body cavity of each eel was opened, and it was examined for abnormalities, in and around the internal organs. A fresh smear of intestinal contents was directly examined for parasites, with a light microscope, like described above.

**Bacteriology**

For bacteriology, specimens were taken with sterile cotton swabs from skin lesions, after they were flambated with a heated scalpel, and cut open with a sterile scalpel. Furthermore, specimens of liver, and kidney were taken with sterile cotton swabs. All specimens were inoculated onto Brain Heart Infusion agar with 5% (v/v) sheep blood. After inoculation the agar plates were incubated at 22°C for maximum 7 days, and were daily examined for bacterial growth. If there was no bacterial growth after 7 days, or, if there was multibacterial growth without colonies looking morphologically like atypical *Aeromonas salmonicida* or *Vibrio* species, the agar plates were no longer incubated. If there was a predominant or pure culture of bacteria from organs and/or skin lesions, the bacteria were typed according to Barrow and Feltham (1993).

**Virus isolation**

In the period 1996-2000 we tested 87 pools of eel organs from disease outbreaks through virus isolations. Depending on the number of eels present, per eel group 5-10 spleens, kidneys, and livers were pooled, and, in case of skin lesions, sterile cotton swab samples were taken from these lesions. Gills were also sampled from at least 4 groups of these fish. Pools of liver, spleen and kidney per eel group, and pooled gills per eel group were separately ground with sterile sand in sterile mortars and pestles. Than, a 10% (w/v) suspension was made of the tissue pool in cell culture medium, containing Earle’s Minimal Essential Medium (EMEM) with 2% foetal bovine serum (v/v), 1% antibiotics (v/v), and 1%
glutamin (v/v), and incubated at 4°C overnight. To each swab specimen 2 ml of the described medium was added, and these tubes were also incubated at 4°C overnight. Next day, the organ suspension was centrifugated twice at 3000 rpm (2000xg) for 10 minutes at 10°C. The supernatants were collected. The swabs were squeezed by pressing them against the tube wall, the suspensions were collected, and centrifuged like described before.

Virus culture medium was made: Leibovitz-L15 medium (Gibco) supplemented with 2% foetal bovine serum, 1% antibiotics (v/v) and 0.075 to 0.15% (w/v) bicarbonate. Two ml of the virus culture medium was added with 0.5 ml of the 10% suspension of each sample to confluent monolayers of 1 day old, in sterile 6 well macroplates (Greiner) of Eel Kidney (EK-1) (Chen et al., 1982) cell lines. These were incubated during 1 h at 26°C in a CO2 incubator. The excess of medium was sucked off. Than, 5 ml virus culture medium was added per well. Cells were incubated for 7 days at 26°C in a CO2 incubator.

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Daily, the plates were checked for cyto pathogenic effect (CPE), with an inverted light microscope. When no CPE was observed after 7 days, the macroplates were frozen at -70°C for at least one night, thawed at room temperature, and the cell lysates were centrifuged for 10 min at 3000 rpm (2000xg) at 10°C, after which the supernatant was examined with an electron microscope.

**Electron microscopy**
A 400-mesh nickel grid with a carbon coated collodion film was placed upside down on a drop of the virus suspension. After 10 minutes incubation, the grids were washed with destilled water and negatively stained with 2% phosphotungstic acid pH 6.8. Grids were examined for presence of viral particles with a Philips CM10 electron microscope.

**Virus DNA isolation and DNA restriction analysis**
For viral DNA restriction analysis, viruses were propagated as follows: 5 ml of the 10% suspension of the isolated viruses from Dutch eels and HVA-Jap (*Herpesvirus anguillae*, isolated from European eel *Anguilla anguilla* in Japan (H. Fukuda, 1999, pers.comm.)), were inoculated on 1 day old monolayers of EK-1 cells in sterile F75 flasks (Falcon) with medium (L-15 +2% fetal bovine serum +1% antibiotics (v/v), and 0.075 to 0.15% (w/v) bicarbonate), which were incubated during 1 h at 26°C. Than, 20 ml medium was added per flask. Flasks were further incubated at 26°C until 100% CPE. Than the flasks were frozen at -70°C overnight, and thawed at room temperature. Viral DNA purification was performed using the procedure described by Hayward et al., 1975.

Purified viral DNA was restricted by *Hind*III under conditions as described by the manufacturer (Boehringer, Mannheim). The DNA molecular marker , λ-DNA, cleaved with *Hind*III (marker II) or cleaved with *Hind*III and...
Eco R1 (marker III) (Boehringer, Mannheim) were used. Gels were stained with ethidium-bromide and photographed using an UV-transilluminator (Land Camera Polariod MP4).

### Results

#### Necropsy and clinical pathology

Some characteristics of the 19 outbreaks from which herpesvirus could be isolated are summarized in Tables 1 and 2. Clinical signs of disease (Table 1) varied, between and also within one farm (for instance outbreaks no. 3 and 4 were at the same farm). Clinical signs in the eels, most frequently seen were apathy, haemorrhages in their skin and fins, ulcerative skin and fin lesions, haemorrhages in the gills, congested gill epithelium, and a pale liver. The eels sizes varied between 9 and 65 cm (Table 2). In outbreak no. 8, HVA was isolated from eels, showing no clinical signs of disease. The mortalities varied, but were lower than 10%. The water temperature at an outbreak was mostly 23-26°C. Most probable stress factors like a bad water quality, or grading of the fish were in most cases the stress trigger for the outbreak.

![Image]

Figure 1 shows eels from an outbreak no. 13 (our ref.no. 519348). In this case there was a mortality of 5%, which occurred at a water temperature at the eel farm of 25°C. The eels showed rough petechial haemorrhages in the skin, with small ulcers all over the body, and a red anus. The gills were also badly affected with congested epithelium and haemorrhages. Internally, this fish showed a pale spleen, liver, and kidney, a congested gall

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Table 1. Predominant clinical signs of 19 disease outbreaks in European eel (*Anguilla anguilla*) at Dutch eel farms, from which HVA was isolated. *Further data per outbreak are presented in Table 2. **Outbreaks at the same farms, respectively: Nos 3 and 4; 10 and 16; 12 and 17; 14, 15 and 19.
bladder, haemorrhages in the mesenteria and muscle, and ascites with blood. Probable stress triggers were the grading of the eels and gas bubble disease.

Figure 2 shows eels from outbreak no. 15 (our ref.no. 534000) with a mortality of 5% at 25-26°C. The eels showed finer petechial haemorrhages in the skin, operculum and belly, and loss of slime from the skin. These fishes showed also pale gills, with congested lamellae and epithelium, and haemorrhages. The spleen was pale and not congested, the liver pale was with haemorrhages, there was ascites and a congested gall bladder. In this outbreak we recommended the eel farmer to decrease the water temperature to 22-23°C. This, in combination with addition of some kitchen salt resulted in a slight decrease of mortality.

Parasitology
Results of parasitological investigation are also presented in Table 2. Wet preparations of skin slime and gills revealed in some cases mostly low numbers of Trichodina sp. and Chilodonella species on the skin, and in some more cases low to high numbers of these parasites, and/or white spot (Ichthyophthirius multifiliis), Dactylogyrus species or Gyrodactylus species.

Bacteriology
Results of bacteriology are presented in Table 2. At the skin the number of bacteria was higher than normal in 6 cases of 19, and consisted of various species, 2 times including myxo-like bacteria. At the gills various species of bacteria were found in 3 cases of 19, but primary pathogenic species were not found. The other cases had no increased numbers of bacteria at the skin and gills. Bacteriological examination of livers and kidneys revealed 7 cases of 19 to be negative for bacteria and 10 cases to consist of opportunistic bacteria like Vibrio species, Pseudomonas species, and Aeromonas species. V.vulnificus + V.harveyi were isolated from 1 case.

Virus isolation
Tissue suspensions from all 19 cases (1 pool per case) caused cytopathic effect (CPE) due to Herpesvirus anguillae in the inoculated EK-1 cells. However, the 4 gill pools (1 pool per case) tested were all positive for HVA, whereas from these 4 cases only 2 pools of
Table 2. General diagnosis (virology, bacteriology, parasitology) and some further parameters of 19 disease outbreaks in Dutch farmed European eels from which herpesvirus (HVA) was isolated. *Outbreaks at the same farms, respectively: Nos 3 and 4; 10 and 16; 12 and 17; 14, 15 and 19. **Dact = Dactylogyrus; Gyrod = Gyrodactylus; Chilo = Chilodonella; Tricho = Trichodina; Costia = Ichthyobodo. *** e/d = eels per day. OTC = oxytetracycline.

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<tr>
<th>Disease Outbreak</th>
<th>Virology</th>
<th>Bacteriology</th>
<th>Parasitology</th>
<th>Other Parameters</th>
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</table>

EVE = European eel, % = percentage, + = positive, - = negative, ND = not determined.

Table 2. General diagnosis (virology, bacteriology, parasitology) and some further parameters of 19 disease outbreaks in Dutch farmed European eels from which herpesvirus (HVA) was isolated. *Outbreaks at the same farms, respectively: Nos 3 and 4; 10 and 16; 12 and 17; 14, 15 and 19. **Dact = Dactylogyrus; Gyrod = Gyrodactylus; Chilo = Chilodonella; Tricho = Trichodina; Costia = Ichthyobodo. *** e/d = eels per day. OTC = oxytetracycline.
liver, spleen, and kidney were HVA-positive. Virus isolations of swab samples of skin ulcers were also found HVA-positive. Virus isolations (Table 2) from organ pools of 4 outbreaks revealed a herpesvirus and another virus in addition (adenovirus, rhabdovirus of eel, birnavirus of eel, and an untyped virus).

**Electron microscopy and virus morphology**
Electron microscopy revealed, that the isolated viruses of the 19 outbreaks all had a typical herpesvirus morphology, and in 4 cases additionally adenovirus, rhabdovirus, birnavirus, and an untyped virus, respectively.

**Virus DNA isolation and DNA restriction analysis**
Gel electrophoresis of virus DNA digested with HindIII showed similar profiles of DNA fragments for all Dutch herpesvirus isolates (Figure 3a). A small difference was observed between Dutch isolates and the Japanese isolate (HVA-Jap), however: the DNA pattern of the Dutch isolates showed 3 fragments, and the Japanese isolate showed 2 fragments in the region above the 23 kilobase pairs.

**Discussion**
In eels, herpesviruses are associated with skin lesions (Kobayashi & Miyazaki 1997), herpes gill filament necrosis (Lee et al., 1999), varicella on the surface of the skin (Ueno et al., 1992) and haemorrhagic septicaemia (Davidse et al., 1999). The herpesvirus isolation from France, described by Jørgensen et al. (1994), was from organ pools of nondiseased European eels. After the first isolation of HVA by Davidse et al. (1999) 18 more portions of eels, of which 17 were diseased, were found positive for HVA. Infected eels showed predomi-
nantly apathy, large and fine haemorrhages in the head, lower mouth, operculum, and fins, skin ulcers, and haemorrhagic and congested gills. At internal inspection, the clinical signs were a pale liver and spleen, haemorrhages in the liver, distension of the gall bladder, and ascites. These signs overlap with those found in South East Asia, where *Herpesvirus anguillae* (HVA) has been isolated from diseased Japanese eel *Anguilla japonica* with 1% mortality, and European eel *A. anguilla* with 6.8% mortality in Japan (Sano et al. 1990), with erythema on the skin and gills, and necrosis of the skin, gills, and liver. Ueno et al. (1992) isolated herpesvirus Formosa (EHVF) from Japanese eels in Taiwan, showing varicella on the skin, mucous secretion and haemorrhages on the gills, swelling of the kidney, distention of the gall bladder, paleness of the liver, and marked enteritis. In Europe, Békési et al. (1986) described mortality associated skin lesions of European eel in Hungary, as necrotic areas of different extent, which developed into patchy regions with hyperaemic halo or into ulceration. Although the description of the skin lesions of European eel by Békési et al. (1986) corresponds with our findings, we cannot state, that it was the same disease, as they did not describe the internal pathology of the eels, nor was the virus isolated. Moreover, the clinical characteristics in our study varied (Table 1). The mortality of maximum 10% we found is slightly higher that Sano et al. (1990) found (6.8%) in European eels with HVA infections.

There were some double infections with *Vibriosis* (2x) and other viruses (4x). Also, secondary bacterial infections (6x) and some ectoparasites were found in this study, apart from HVA. The predominant lesions of Table 1, however, were found at least in outbreaks from which only the virus HVA was isolated.

In 2 cases of our study, virus isolations of organ pools were negative, but of homologous gill pools positive. Skin ulcers were also found HVA positive. It is therefore recommended, to sample at least gills and skin ulcers for HVA isolations of eel, apart from the internal organs. The gills might act as the porte d’entree for HVA. This should be further investigated.

Although Anders & Yoshimizu (1994) described the relation of herpesviruses and other viruses to skin tumours and tumour-like proliferations in fish, in eels only epidermal hyperplasia, related to herpesvirus was found, like described by Békési et al. (1986). Several authors have proven, that some fish herpesviruses have oncogenic properties (Kimura et al. 1981a, 1981b; Sano et al. 1983, 1985). We did not detect any tumour-like lesions on and in the diseased eels, nor did we yet investigate, if our isolate has oncogenic properties. More investigations, like pathogenicity studies with histopathology should be done.

The DNA restriction patterns of viral DNA of our Dutch herpesvirus isolates were similar to that of *Herpesvirus anguillae* (HVA-Jap), but showed some differences, after treatment of the DNA with *Hind*III. Comparison of DNA from various HVA isolates can be helpful to reveal genetic relationships. It would be interesting, to compare our virus isolate (486123) with EHVF (Ueno et al. 1992), and with the French isolate, described by Jørgensen et al. (1994).

The water temperature was 23-26°C in 11 HVA outbreaks of our study. Lowering the water
temperature induced a decrease of the eel mortality in some cases (pers. communication with eel farmers). The mechanism behind this is yet unclear. It could be an immunological mechanism, as HVA replication in vitro is not inhibited much at 20°C compared to 26°C (Van Nieuwstadt, unpublished). It should be further investigated, if lowering the water temperature helps to control the disease at eel farms. Also, the incubation time of HVA infections needs to be determined.

Herpesvirus is most probably indigenous to the wild eel population (Haenen, unpublished results). Disease caused by HVA infection may have become manifest by culturing eels in high stocking densities, which means stress. During the last 20 years culture of eels in recirculation systems using bio filters for water purification has expanded in the Netherlands. It implicated initially much contact (man, restocking eels etc.) between eel farms, and the use of wild restocking eels (unpublished data). This practice of eel farming will have supported the spread of herpesvirus among cultured eels, and so a possibly endemic HVA infection which passed unnoticed among wild eels may have caused complications in farmed eels.

Since when is HVA present in Europe, and how far is it spread? Sofar no reports about the use of one of the susceptible cell lines are known, apart from the use of RTG-2 and FHM cell lines by Békési et al. (1986), and RTG-2 and EPC cell lines by Jørgensen et al. (1994), which were only partly susceptible, in last paper. Diseased European eels from all European countries should be checked for herpesvirus to investigate the epidemiology of the virus. Also, elvers need to be checked to prevent spread of HVA.

The herpesviruses, described by Békési et al. (1986) and from this study obviously are related to disease of European eel. Pathogenicity studies with elvers and bigger European eels need to be done to estimate the risk of the Dutch herpesvirus isolate for European eels, and if it is very pathogenic, also for other cultured fish species. In this study stress was mostly present, at the start of the outbreak. There is always some stress at eel farms. The role of stress is important, to induce replication of HVA (Van Nieuwstadt et al., 2001), after which disease may develop.

Further investigations include development of a PCR for HVA, the epidemiology of HVA in Europe, pathogenesis and pathogenicity studies (Basavarajappa et al., to be published), and molecular typing of Dutch and foreign herpesvirus isolates (Van Nieuwstadt et al., to be published). Pathogenicity studies in other fish species could also be done, but only, if the virus appears to be pathogenic to European eels after experimentally induced infections. Prevention of HVA infections could be practiced by testing restocking eels, elvers, and farms for absence of HVA by virus isolations, and isolating the HVA free farm from possible HVA positive sites in the outside world. Vaccination is no option, as HVA is a secondary cause of disease, after a stress trigger.

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References


