First confirmation of Herpes Virus Anguillae (HVA) and Infectious Pancreatic Necrosis (IPN) virus infecting European eels *Anguilla anguilla* farmed in Greece

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Abstract

For several years eel farms situated in NW Greece, kept reporting, lack of appetite and alarming losses among growing eels of various size classes. The problems usually occurred subsequent to handling, such as grading. During the recent years 2008-2010 these problems intensified. Necropsy, bacteriology and parasitology performed on diseased specimens often resulted in mixed findings, such as *Aeromonas* spp. infections or *Dactylogyrus* spp., *Trichodina* spp. and *Myxidium* spp. infestations, none of which was deemed sufficiently intense to explain the severity of symptoms and the persistence of losses. Antibiotic treatments and/or antiparasitic baths did not help. Common lesions comprised gill inflammation and often bleeding of the gills and red sore like dermal lesions around the head and opercular regions. The pectoral fins were frequently haemorrhagic. These lesions and the frequency and persistence of morbidity suggested the possibility of infection by Herpes Virus Anguillae (HVA). Frozen samples at -20°C of diseased whole eels of three different batches from the same farm were collected in spring and summer of 2010 and sent to a virology laboratory. Virus isolations were performed on eel kidney (EK-1) and Bluegill fry (BF-2) cell lines, where cytopathic effects were observed. Both the IPNV and the HVA agents were isolated and identified by IFAT and the viral particles were observed by transmission electron microscopy. This is the first published report of HVA and IPN viruses infecting farmed eels in Greece.

Introduction

Most eel farms in Greece are situated in the north-west region of the country at the northern banks of the Amvrakikos gulf where the Arachthos and Louros river deltas form a suitable estuarine environment to grow euryhaline fish species, such as grey mullets, but also provide ample fresh water of good quality to grow fresh water species such as trout. Farmers take advantage of these water resources, some growing more than a single species, either intensively in concrete raceways, or semi-intensively in earth ponds or lagoons. European eels (*Anguilla anguilla*) as well as rainbow trout (*Onchorhyncus mykiss*) are occasionally grown on the same farm.

Raceways are supplied by riverine or borehole fresh water, held in a reservoir. Water recirculation systems are in place for indoor elver tanks.

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Water temperature may range between 18°C and 24°C. The water from the raceways is returned to the river or estuary untreated. Trout eyed ova and glass eels or elvers are imported for hatching and ongrowing.

Perennial disease problems that are responsible for considerable losses among the eel populations are caused by bacteria, most often by the motile aeromonads *Aeromonas hydrophila* and *A. sobria* and parasites, the most important among which is the gill fluke *Dactylogyrus spp.*, followed by the myxosporean *Myxidium spp.* and the gill ciliate *Trichodina spp.*

Since 1998, about 20 disease outbreaks at two adjacent eel farms have been reported to a private veterinary laboratory in Athens and samples were submitted for diagnostic investigation. Although bacteria and parasite pathogens were diagnosed in most cases, the gross clinical signs, high morbidity and rather low mortality patterns suggested an underlying viral aetiology. In particular, the disease signs commonly associated with HVA infection matched the most abundant lesions and the repetition of outbreaks. During the summers of 2009 and 2010, a farm reported much more severe and persistent outbreaks with widespread morbidity and cumulative mortality around 15% mainly among the younger eels. Morbidity and mortalities were seen to rise subsequently to handling, such as grading or transportation from the indoor nursery tanks to the outdoor raceways for ongrowing.

**Materials and methods**

**Fish samples**

Moribund European eels belonging to three batches, ranging in size from 12g to 80g average wet weight, were collected from a farm during two outbreaks in the spring and summer of 2010. The batches originated from different elver imports. The water temperature was approximately 23°C in both occasions. The eels were transported on ice in insulated boxes to the private VetCare™ laboratory in Athens, Greece. They were delivered approximately 24 h after sample collection. A few specimens were still alive at delivery to the laboratory and were placed in an aquarium with tap water for observation. These eels succumbed naturally after four h and then underwent post mortem necropsy. The internal organs were examined for the presence of gross lesions and then submitted for bacteriological examination. Fresh preparations of skin, gills, intestines, kidneys and swim bladders in the form of smears, scrapes, and/or squashes were examined microscopically for the presence of parasites. Three randomly selected eels from each batch (n = 9 in total), were frozen at -20°C and stored until transportation by air courier in a special cool-box for medical samples to the Istituto Zooprofilattico Sperimentale delle Venezie, Padova, Italy, for virological examination. They were delivered the following day. Sample group S1 was frozen on April 21st 2010 and comprised 3 eels with body weights: 44g, 55g, and 74g. Group S2 was also frozen on April 21st 2010 and comprised 3 eels with body weights: 13g, 18g, and 18g. Sample group S3 was frozen on June 1st 2010 and comprised 3 eels with body weights: 17g, 24g, and 26g. In order to reduce total sample weight and volume for transport, the rear third of the body, posterior to the hind kidney, had been cut off.

**Bacteriological investigations**

Inocula from the liver, spleen and kidney of
the eels were taken with flame sterilized loop and plated onto Tryptone Soya Agar (TSA; Biomerieux™, France). The cultures were incubated at 25°C for 36 h. Developing colonies were examined by Gram stain and biochemical reactions performed on API-20E test strips (Biomerieux™, France). The isolates were tested for resistance to selected antibiotics.

**Virological investigations**

Samples S1, S2 and S3, each one consisting of three animals showing HVA lesions, were immediately processed for virological examination upon receipt. From each group two different sub-samples were prepared. In the first, kidney, spleen and heart were pooled (sample identification P-S1, P-S2 and P-S3) while in the second sub-sample only gills from the same fish were included (sample identification G-S1, G-S2 and G-S3). Six samples were processed in total.

Following homogenization by mortar with sterile sand, each pooled sample was suspended 1:10 in Eagle’s Minimum Essential Medium (EMEM), supplemented with 10% v/v fetal calf serum (FCS) and 2% v/v of antibiotic-antimycotic solution (Penicillin 100 UI/ml, Streptomycin sulphate 10mg/ml, Amphotericin B 25μg/ml and Kanamycin 10 mg/ml.) (Sigma Aldrich, USA).

Tissue extracts were centrifuged at 3,000 x g for 30 min and incubated over-night at 4°C. Eel kidney (EK-1) (Chen and Kou, 1982), Bluegill fry (BF-2) (Wolf and Mann, 1980) and epithelioma papulosum cyprini (EPC) (Fijan et al., 1983) cell lines were grown in 25cm² tissue culture flasks (BD Bioscience, USA) in cell culture medium EMEM supplemented with 10% FCS, 1% L-glutamine, 1% antibiotic-antimycotic solution without Kanamycin (Sigma Aldrich, USA) at 25°C. Tissue extracts were inoculated at two tenfold dilutions (1:10 and 1:100) onto 1-day-old EK-1, BF-2 and EPC cells grown in 24-well cell culture plates (BD Bioscience, USA) and incubated at 20°C. After inoculation, plates were observed daily for detection of cytopathic effect (cpe). After 7 days supernatants were filtered through 0.45 μm membranes and used to inoculate actively growing EK-1 and BF-2 cells. Samples were examined along one progressive passage of 7 days.

**Immunofluorescence**

The supernatants from cpe positive monolayers and the HVA reference virus (strain 500138, kindly provided by Dr. Olga Haenen, Central Veterinary Institute, The Netherlands) were inoculated onto 1 day-old EK-1 monolayers grown on cover slips (Thermo Scientific, USA) placed in 24-well plates and incubated at 25°C. When the cpe was clearly visible (24-48h) the medium was removed and the monolayers fixed with 80% acetone in PBS (prepared in the laboratory) for 10 min. Fixed cells were treated with 0.2 ml/well of rabbit polyclonal hyperimmune serum (anti-HVA-839 diluted 1/1000, kindly provided by Dr. Olga Haenen, Central Veterinary Institute, The Netherlands). Distinct monolayers inoculated with the same samples were treated with in-house produced rabbit polyclonal hyperimmune serum (anti-IPNV-Ab, Sp, VR299), diluted 1/500. Hyperimmune sera were obtained from SPF rabbit (Oryctolagus cuniculus) after subcutaneous inocula according to antisera production schedule described by Douglas et al. (1985). After rinsing, cells were stained with 0.2 ml goat isothiocyanate-fluoresceinated anti-rabbit Ig (SIGMA Aldrich). The glass slides were finally observed under incident UV light using
a fluorescence microscope (Zeiss Axioscop) equipped with an epifluorescence system (Short Arc Mercury Lamp, HBO 100 W).

**Transmission electron microscopy (TEM)**

Aliquots from tissue homogenates prepared for virus isolation were processed for TEM investigations.

They were centrifuged 30 min at 2750 x g at 8℃. 120 μl were ultracentrifuged (rotor A-100, Beckman Airfuge) for 15 min at 20 psi (100,000 g) to pellet the viral particles on formvar carbon coated copper grids (Doane and Anderson, 1987). The grids were stained using a 2% sodium phoshotungstate solution (Electron Microscopy Science, USA) in distilled water (pH 6.8) for about 3 min. The dried grids were observed using a Philips TEM operating at 80kV, at 19,000-45,000x magnification.

Supernatants from cell cultures showing clear cpe were repeatedly frozen and thawed and processed in the same way as for tissues.

**Results**

**Gross clinical signs**

The diseased eels were off feed for several days, they appeared weak and lethargic and their skin was dark in colour. No muscle emaciation was evident. Common lesions comprised haemorrhagic dermal ulcers on the head, especially located on the opercular skin, lips and lower jaw. The pectoral fins were inflamed, haemorrhagic and often eroded. A bloody exudate was seen to come out of the gill cavity due to severe gill congestion and exsanguinations of blood (Figure 1). The gill epithelium was congested and inflamed with increased mucus secretions among the gill filaments, however, no necroses were evident (Figure 2). There was no exophthalmia or distention of the abdomen, but on some eels the abdominal skin was mildly haemorrhagic.

Regarding the internal organs, the only suspect findings comprised congestion of the kidney and macroscopic signs of hepatic inflammation, such as congestion and petechiae. There were no lesions on the heart, spleen, gall bladder and swim bladder. The alimentary tract was empty of food or feces and the intestinal mucosa did not show signs of haemorrhage. The peritoneal hymens, mesentery and pericardium were normal.

Microscopic examination of gills revealed a mild parasitic infestation by monogenetic trematodes of the genus *Dactylogyrus* as well as by protozoan ciliates of the genus *Trichodina*. The presence of bacteria on the epithelium and in the mucous excretions was low.

**Bacteriological investigations**

Examination of bacterial cultures on TSA did show some limited growth of Gram negative bacteria colonies, which were pale, round and 1-2mm in diameter. These bacteria gave an API-20E profile of 3247124, which identifies the genus *Aeromonas spp.* and were found resistant in vitro to ampicillin, amoxycillin, slightly sensitive to potentiated sulfonamides and oxytetracycline and sensitive to flumequine and oxolinic acid.

**Virological investigations**

A clear cpe was observed starting two days after the second subculture on both BF-2 and EK-1 cell lines while no cpe appeared on EPC cultures, even by the end of the second blind
Figure 1. European eel (*Anguilla anguilla*). Haemorrhagic inflammation of the pectoral fins and bloody exudate coming out of the gill cavity (arrow) due to gill congestion and exsanguinations of blood.

Figure 2. European eel (*Anguilla anguilla*). Congested gills without necrosis of the gill filaments.
passage. In EK-1 cultures, a progressive detachment of the cells resulting in complete monolayer destruction was evident in all the samples except for sample G-S3 in which clear syncitia were observed (Figure 3).

The second blind passage was performed from EK-1 monolayers 7 days post inoculation. Following the second passage, a clear cpe developed after 3 days both in BF-2 and EK-1.

**Immunofluorescence**

Samples P-S1, P-S2, P-S3, G-S1 and G-S2 tested positive by IFAT for IPN virus on EK-1 and BF-2 infected cells, while sample G-S3 tested positive for HVA on EK-1 infected cells. IPNV positive samples showed the presence of single fluorescent cells (Figure 4) scattered into the cell cytoplasm and characterized by intensively stained globules as previously described by Donald et al. (1973). The IFAT performed

![Image](image_url)

**Figure 3.** A. EK-1 cell culture showing syncitia cpe (arrows) due to HVA infection. B. EK-1 negative control.
on the HVA positive sample G-S3 showed the presence of brilliant fluorescent single cells and typical syncytia characterized by intracellular inclusions (Figure 5).

**Transmission electron microscopy (TEM)**

Round particles, of 100-110 nanometers in diameter with concentrically located electron-opaque rings and electron-dense nucleocapsids, resembling Herpesvirus virions (Figure 6), were observed in all three samples prepared from pooled gills (G-S1, G-S2 and G-S3).

Samples from pooled tissues (kidney, spleen and heart) and supernatants obtained from BF-2 cell culture infected with the same pools of tissue revealed the presence of Birnavirus-like particles (icosahedral particles of 50-60 nanometers in diameter, results not included).

HVA detected on EK-1 was not tested with TEM.

**Discussion**

This report represents the first isolation of HVA associated with IPNV from farmed European eels in Greece and could explain the persistent perennial morbidity observed in eel stocks subsequent to handling stress without the detection of serious parasitic infestation or bacterial infections.

HVA is the causative agent of a serious disease in the European eel industry and has been iso-
Figure 5. Positive HVA immunofluorescent staining on infected EK-1 monolayer from eel sample GS-3.
lated from wild eels (Ginneken et al., 2004), confirming that the virus is widespread.

The disease is characterised by hemorrhages in the head, lower mouth, operculum, fins, skin ulcers and haemorrhagic and congested gills (Haenen et al., 2002). The lesions observed in our study fully correspond to those observed in European eels farmed in The Netherlands (Haenen et al., 2002), in Japanese and European eels farmed in Japan (Sano et al., 1990) and in Taiwan (Ueno et al., 1992) as well as in Hungary (Bekesi, 1986).

Different assumptions could be made as regards to the origin of HVA and/or IPNV. Eel farming in Greece is based on imports of young eels; hence, it is possible that the diseases had been imported with latently infected eels. HVA persists for a long time in clinically healthy hosts which have been previously exposed to the virus (Niewstadt et al., 2001) and under stressful conditions may be activated and shed, without clinical symptoms, infecting naive eels. Alternatively, there is the possibility that the viruses were already present in the farm infecting imported young elvers.

Concerning the detection of IPNV, previous investigations carried out on diseased trout coming from other farms in the same region, which originated from Danish egg imports, confirmed IPNV belonging to the A2 serotype (Varvarigos and Way, 2002). In the present study...
the isolated IPNV was not characterised.

In this outbreak HVA was isolated only from gill samples. This finding is partially in agreement with the results obtained by Haenen et al. (2002) and suggests that the virus is mainly replicating in this tissue. In fact, all three corresponding samples obtained from pooled heart, spleen and kidney tested negative. The IPN virus has been isolated also from pooled tissues including kidney. Kidney seems to be one of the most suitable organs for IPNV diagnostic purposes in fish (Yamamoto, 1975).

Although Herpesvirus-like particles were observed by TEM in all gill samples, only one sample tested positive for HVA with IFAT. It could be that IPNV growth reduced the possibility of HVA detection.

The detection of both IPNV and HVA has already been reported in elvers of 20-40 g (Haenen et al., 2002) reared at the same temperature (22°C). In that outbreak the mortality rate was lower. In this study it was not possible to determine which virus assumed prime responsibility for mortality. Experimentation under controlled conditions would be necessary in order to define this.

In order to implement disease control measures in the affected region and prevent re-infections of either HVA or IPN diseases it would be important to assess the magnitude of the disease spread by screening wild fish as well as farmed populations for the presence of seropositive fish. Nevertheless, neither state intervention nor coordinated actions by the farmers are expected, hence, HVA together with IPN have to be considered in the future as serious causes of economic loss.

References


