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Virus infections of the European Eel in North Rhine Westphalian rivers

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Abstract

Viral infections have been suggested to play a role in the decline of the panmictic population of the European eel (*Anguilla anguilla*). However, despite the importance of knowledge about pathogenic eel viruses, little is known about their spread in the wild European eel population and only a few eel pathogenic viruses have been described so far. In this study, we aimed to investigate the health status of the *A. anguilla* stock in North Rhine Westphalia (NRW) State of Germany. For this purpose, we examined tissue samples of 16 elvers, 100 yellow eels and 6 silver eels, sampled from the rivers Rhine, Lippe and Ems. Virus detection was performed via a combination of cell culture and PCR. Next to the detection of frequently encountered pathogenic eel viruses (anguillid herpesvirus 1 and eel virus European X (EVEX)), we isolated the eel picornavirus 1 (EPV-1) from tissue of yellow eels and elvers and demonstrate the distribution of EPV-1 in wild eel population in NRW.

KEYWORDS

Anguilla anguilla, anguillid herpesvirus 1, eel picornavirus 1, eel virus European X, European eel

1 | INTRODUCTION

The panmictic population of the European eel *Anguilla anguilla* is declining dramatically throughout its distribution range since the end of the 1970s (Correia et al., 2018; Feunteun, 2002, ICES, 1999). The number of eels caught in Europe has decreased by more than 75%, and spawning stock biomass is estimated to range from 2% to 12% of its former range. Furthermore, the glass eel recruitment has dropped to only 5% of the mean values throughout the distribution area and less than 1% for the North Sea recorded from 1960 to 1979 (ICES, 2012). *Anguilla anguilla* has developed a complex but sensitive lifecycle that includes long-distance migrations which makes them susceptible to anthropogenic pressure or changed environmental factors. Thus, a combination of detrimental factors might have caused this tremendous decline (ICES, 2012).

Gulf Stream shifts are considered to reduce the survival of leptocephalus larvae during their transoceanic migration (Feunteun, 2002). Overfishing of all life stages, habitat loss and decreasing water quality in the past decades might further affect the European eel population (Geeraerts & Belpaire, 2010). The blockage of migration routes by weirs and dams and especially the turbine losses by hydropower plants further contribute to the decline (Castonguay et al., 1994). However, the complex correlation of these factors is not fully understood yet (Dekker, 2003). In addition, it is discussed whether the spread of infectious diseases might contribute to reducing spawner escapement from European inland waters to the Sargasso Sea (Haenen et al., 2009; van Beurden et al., 2012; van Ginneken et al., 2004). Besides infections with the well-studied but still harmful parasitic swim bladder nematode *Anguillicola crassus* (Kirk et al., 2000), particularly virus infections are discussed to play

a contributory role in the decline of the European eel (van Ginneken et al., 2004, 2005).

To date, only a few pathogenic eel viruses have been described. The alloherpesvirus anguillid herpesvirus 1 (AngHV-1) is well characterized and its distribution in different countries in Europe has been investigated. The first isolation and characterization of AngHV-1 were performed in 1985 by Sano et al., 1990. AngHV-1 was isolated from diseased *A. anguilla* and *A. japonica*, which were farmed in Japan using the eel kidney 1 (EK-1; Chen et al., 1982) cell line. In addition, AngHV-1 was detected by PCR in *Anguilla rostrata* (Kempter et al., 2014; Shih, 2004) and *A. marmorata* (Panicz et al., 2021). AngHV-1 infections can result in chronical infections and high mortality rates (van Nieuwstadt et al., 2001). The outbreak of AngHV-1 infections is temperature dependent, and clinical signs are most obvious at 25–26°C. Further, it is assumed that the disease outbreak is triggered by stress conditions (Haenen et al., 2002). In the following years, AngHV-1 was detected in wild-caught *A. anguilla* and *A. japonica* (EFSA, 2008; van Beurden et al., 2012). In 1998, this herpesvirus was detected in European eels caught in Europe in the Netherlands for the first time (Davidse et al., 1999; Haenen et al., 2002). An extensive study of the distribution of AngHV-1 in Schleswig-Holstein (Germany) revealed an infection rate of 68% in the river Schlei (Kullmann et al., 2017). However, knowledge about the distribution of eel viruses in other areas of Germany is still missing.

Another common pathogenic eel virus is the rhabdovirus eel virus European X (EVEX) which was isolated in the 1970s from *A. anguilla* (Sano et al., 1977). An infected elver was caught in France. A similar rhabdovirus was detected in *A. rostrata*, which was designated as eel virus American (EVA) (Sano, 1976). Both viruses share similar morphology as well as serological and physicochemical characteristics (Hill et al., 1980; Nishimura et al., 1981). However, EVEX and EVA were classified as two distinct species (Hill et al., 1980; Nishimura et al., 1981; Stone et al., 2013; van Beurden et al., 2011). Studies in the 1980s revealed a number of EVEX-positive yellow eels in Germany (Ahne et al., 1987; Ahne & Thomsen, 1985) as well as EVEX-carrying elvers (Shchelkunov et al., 1989). For diseases caused by EVEX or AngHV-1 infections, diverse clinical signs have been described (Haenen et al., 2009; van Beurden et al., 2012). Eels infected with these viruses developed haemorrhage and anaemia, for example (Kobayashi & Miyazaki, 1996). In swim tunnel experiments, EVEX-infected eels died after 1.000–1.500 km demonstrating the impact of their migration to the spawning area (van Ginneken et al., 2007).

The isolation of an eel pathogenic aquabirnavirus eel virus European (EVE) has been described in 1976 (Sano, 1976). To date, little is known about the distribution of EVE in the wild eel population in Europe. EVE is the causative agent of the branchionephritis in eels (Okamoto et al., 1983; van Beurden et al., 2012), and EVE infections can cause high mortality rates up to 100% (Chen et al., 1985).

Further, in 2013 a publication demonstrated the infection of an eel caught in Lake Constance with a picornavirus (Fichtner et al., 2013). The virus was designated as eel picornavirus 1 (EPV-1). Besides the infection with EPV-1, the diseased animal carried AngHV-1. Infection experiments demonstrated a mortality rate of 43%. The identification of EPV-1 was performed by next-generation sequencing. Furthermore, orthomyxovirus-like particles were isolated from European eels (Haenen et al., 2009; van Beurden et al., 2012) as well as reovirus-like particles (Haenen et al., 2009). In 2014, circovirus infections of adult European eels have been reported (Doszpoly et al., 2014). Recently, circovirus-infected eels were detected in Hungary. However, the investigated animals did not exhibit clinical signs (Borzák et al., 2017).

In this study, we designed a virus monitoring programme to investigate the distribution and prevalence of AngHV-1, EVEX and EPV-1 in the rivers Rhine, Lippe and Ems in North Rhine Westphalia (NRW). For this aim, cell culture and molecular techniques were combined and RT-(q)PCR methods for the detection of EVEX and EPV-1 were established.

2 | MATERIAL AND METHODS

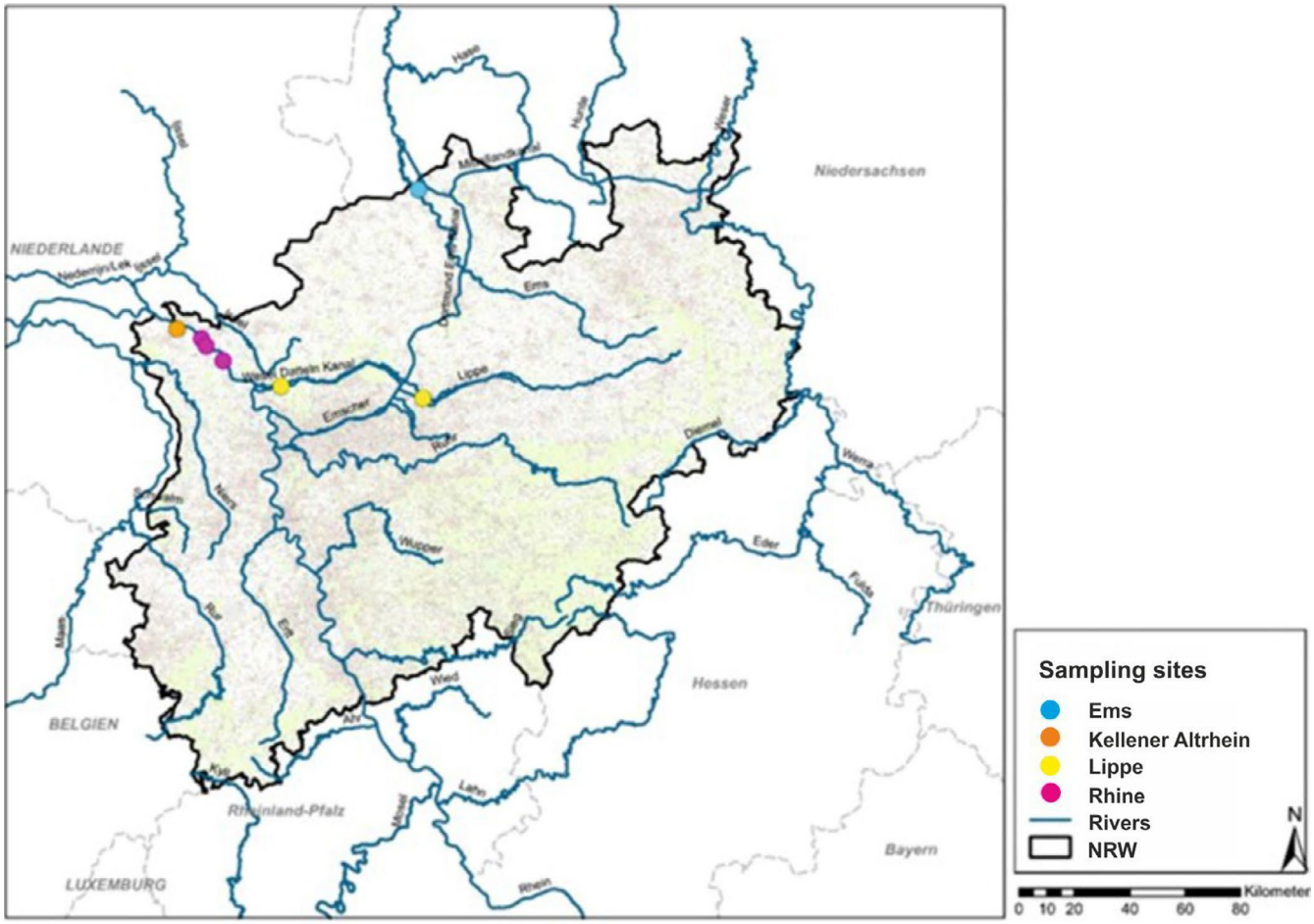
2.1 | Eel catch and sample preparation

A total of $n = 122$ wild European eels were caught from June to October 2018 in different sampling sites (rivers Lippe, Ems and Rhine and the inlet Kellener Altrhein, in NRW, Germany (Figure 1)). Elvers were caught by using elver channels, yellow eels via fish traps and silver eels by specialized fish trap boats. Mapping was performed using the software geographic information system (GIS). Life stages were calculated according to Durif et al. (2009). Sampling sites and collection dates are shown in Figure 1. Animals were kept for 24 h to induce a stress response to enhance the chances of virus detection. Tissue samples (pools of spleen, heart, gills, kidney) were taken from each individual and stored at −80°C for virus isolation via cell culture.

For diagnosis of bacterial infections, samples of spleen, kidney and liver were pooled and inoculated on Columbia sheep blood agar (Oxoid), trypticase soy agar plates (Oxoid) or Cytophaga agar and incubated at 15°C and 20°C. Bacterial growth was monitored for 7 days.

2.2 | Virus isolation

Virus isolation and propagation were performed using the eel kidney 1 (EK-1; Chen et al., 1982) cell line. EK-1 cells (CCLV Rie 809) were provided by the Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Germany. The EK-1 cells were propagated in Leibovitz L-15 medium (10% foetal bovine serum; 100 µg/ml tylosin; 100 µg/ml streptomycin/spectinomycin) in cell culture flask for confluent monolayer at 20°C and 26°C. For testing, organ samples were prelysed using a Tissue Lyser (Qiagen). The homogenate was resuspended in cell culture medium, and samples were centrifuged to remove cell debris (15 min; 2360 x *g*; 10°C). The inoculation



Sampling site	Sampled by	Date of sampling	Caught animals (n)
Ems	LANUV NRW	10.07.2018	31
Lippe	LANUV NRW	17.04.2018	11
Lippe	LFV Lippe	01.06.2018	11
Lippe	Professional fisherman	27.07.2018	9
Rhine	Professional fisherman	22.06.2018	11
Rhine	Professional fisherman	14.07.2018	11
Rhine	Professional fisherman	08.10.2018	12
KellenerAltrhein	Professional fisherman	29.05.2018	16
Rhine	Professional fisherman	October 2018	10

FIGURE 1 Sampling sites of elvers, yellow eels and silver eels; sampling dates are depicted below. LANUV NRW, State Agency for Nature, Environment and Consumer Protection, Fisheries Ecology and Aquaculture, Germany. LFV, Landesfischereiverband Westfalen und Lippe e.V

of EK-1 cells with cleared homogenate occurred at 20°C and 26°C; passaging was performed over 7 days. In total, three passages were performed. Samples showing distinct changes in cell morphology (cytopathic effects (CPEs)) compared with the negative control were defined as virus-positive cell cultures. Negative defined samples were comparable in morphology to mock-infected cells. Screening for CPEs was carried out every 24 h using a Primovert microscope (Zeiss).

Virus identification in cell culture supernatants was performed via PCR-based techniques. Total DNA and RNA were extracted from 100 µl of the supernatant from positive cell cultures in accordance with the manufacturer's instructions using the *Mini kit for DNA from cells and tissue* (Macherey & Nagel) and the *Mini kit for RNA purification* (Macherey & Nagel), respectively. Quality and quantity of DNA and RNA isolates were assessed using the NanoDrop Onec (Thermo Fisher). Virus isolates of AngHV-1 (LANUV NRW 2014), EVEX (Olga

Haenen, Wageningen University & Research) and EPV-1 (LANUV NRW; FLI 2017) were used as positive controls. Virus strains are listed in Table S2. Mock-infected cells were used as negative control.

2.3 | Real-time PCR

The detection of AngHV-1 was performed according to a modified protocol published initially by Rijsewijk et al. (2005) using the SensiFast™ High Resolution Melt (HRM) kit (Bioline) containing EvaGreen as intercalating dye. Primers are listed in Table S1. The final reaction mixture contained a total volume of 20 µl consisting of 10 µl SensiFast™ High Resolution Melt (HRM) kit, primers at a final concentration of 400 nM and 4 µl of template DNA (10–30 ng/µl). The thermal profile consisted of an initial polymerase activation (3 min 95°C); PCR cycling (5 s 95°C, 10 s 65°C, 15 s 72°C); and high-resolution melting curve (65°C–95°C). The PCRs were performed in an Eco Real-Time PCR System (Illumina). Non-template control (water control) and isolated DNA from mock-infected cells were used as negative control.

2.4 | Reverse transcription PCR (RT-PCR)

Detection of EPV-1 and EVEX was performed by RT-PCR using the One-Step RT-PCR Kit (Qiagen) in accordance with the manufacturer's protocol. The reaction mixtures (50 µl) contained 2 µl enzyme mix, 10 µl buffer and dNTPs and primers (0.15 µl) in a final concentration of 400 and 0.6 µM, respectively. The reactions were supplemented with 2 µl template RNA (10–30 ng/µl). For the reaction mixtures, H_2O (Molecular grade, nuclease-free water; Thermo Fisher) was used. RT-PCR was performed in a Mastercyler Pros S (Eppendorf).

The 2C gene was selected as a target sequence for the identification of EPV-1 with a resulting amplification product of 330 bp. Primers deduced from published EPV-1 sequence NC_022332.1 (Fichtner et al., 2013) are listed in Table S1. The thermal profile included the reverse transcription at 50°C for 30 min, an initial denaturation at 95°C for 15 min, followed by 39 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s and elongation at 72°C for 60 s. The rhabdovirus EVEX was detected via amplification of a 482-bp fragment of the L gene. Primers were deduced from published sequence NC_022581.1 (Galinier et al., 2012) and are listed in Table S1. The thermal programme included the reverse transcription at 50°C for 30 min, an initial denaturation at 95°C for 15 min, followed by 39 cycles of denaturation at 95°C for 30 s, annealing at 53°C for 30 s and elongation at 72°C for 60 s.

In both cases, non-template control (water control) and isolated RNA from mock-infected cells were used as negative control. PCR products were purified using the ExoSAP-IT Express kit (Thermo Fisher) according to manufactures instructions and verified via Sanger sequencing (CEQ8000 Beckman Coulter) using the DTCS Quick Starter Kit (Beckman Coulter) according to manufacturer's instructions.

TABLE 1 Eel life stages determined by catching method and according to Durif et al., 2009

Life stage	Ems	Lippe	Rhine	Total
Elver	0	0	16	16
Yellow eel	31	31	38	100
Silver eel	0	0	6	6

3 | RESULTS

In this study, the health of 122 European eels of different life stages was investigated in the rivers Rhine, Lippe and Ems. Based on the sampling method, 16 fishes were designated as elvers. According to Durif et al., 2009, 100 of the eels caught were yellow eels and six were silver eels (Table 1). All animals did not exhibit clinical signs of a disease (data not shown). Besides identification of pathogenic eel viruses via cell-based and molecular techniques, bacterial infections have been investigated. None of the animals suffered from bacterial infections, investigated via cultivation of agar plates (data not shown).

The amount of virus-positive animals divided according to stage of development is shown in Figure 2. 25% of the investigated elvers ($n = 4/16$) originating from the Kellener Altrhein were virus positive in cell culture. Yellow eels were caught in three different locations (Ems, Lippe, Rhine); in total, 54% of these animals carried virus infections which could be detected in EK-1 cells ($n = 54/100$; *of this Ems ($n = 10/31$), Lippe ($n = 16/31$) and Rhine ($n = 28/38$)*). In addition, virus-positive silver eels were detected in the Rhine (83%; $n = 5/6$). The viruses were identified by PCR-based methods. Genomic identification of EVEX was performed by amplification of a 482-bp fragment of the L gene (Figure S1a,b). A 330-bp fragment of the 2C gene was used as a target to identify EPV-1 (Figure S1). The PCR analysis of the elver sampling revealed that none of the elvers originating from the Kellener Altrhein carried AngHV-1 or EVEX infections (Figure 3; Figures S2–S6; Figures S7 and S9). Yet, 18.8% were EPV-1-positive (Figures S8 and S10).

Additionally, virus isolates from yellow eel samples were investigated by PCR. The yellow eel sampling contained 11.0% AngHV-1-positive eels ($n = 11/100$; Figures S2–S6), 22.0% ($n = 22/100$; Figures S7 and S9) EVEX-positive eels and 16.0% EPV-1-carrying animals ($n = 16/100$; Figure 4; Figures S8 and S10). In addition, silver eels caught in the river Rhine were EVEX-positive (67%; $n = 4/6$; Figure 5; Figures S7 and S9). None caught silver eel carried AngHV-1 or EPV-1 infections (Figures S2–S6; Figures S8 and S10). Several eels carried coinfection; the results are summarized in Figure 6:4.9% ($n = 6/122$) were positive to AngHV-1 and EVEX, none carried AngHV-1 and EPV-1 in combination. Further, 4.1% ($n = 5/122$) carried EVEX and EPV-1. 0.8% ($n = 1/122$) were infected with all three viruses.

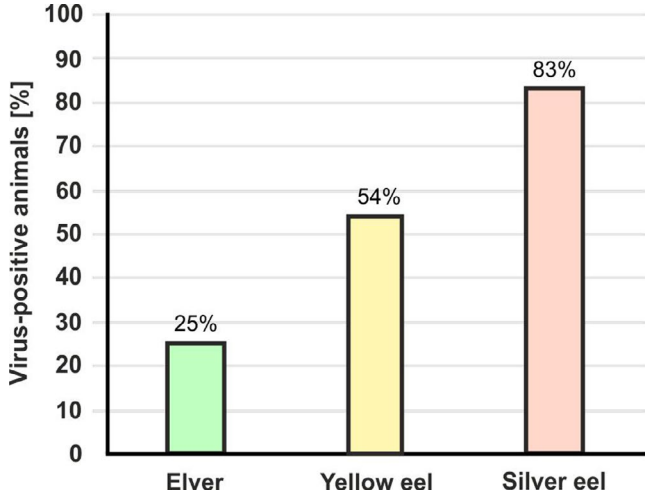


FIGURE 2 Number of wild animals with positive virus detection via cell culture. Elvers (25%, $n = 4/16$); yellow eels Ems (54%; $n = 54/100$); silver eels (83%; $n = 5/6$)

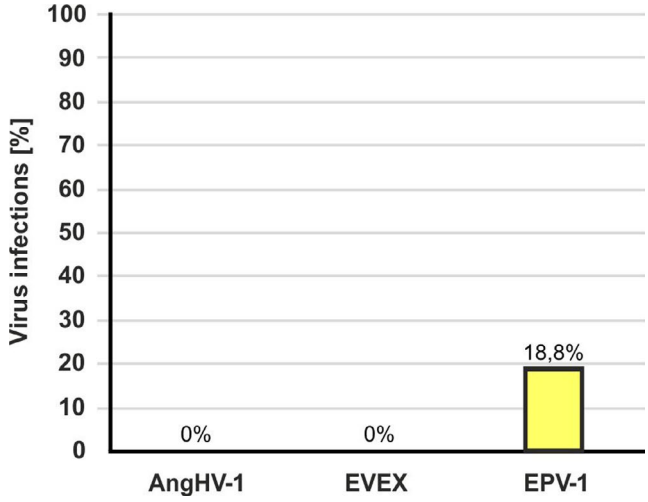


FIGURE 3 Identification of eel pathogens in virus isolates of elvers originating from the river Kellener Altrhein via PCR. (AngHV1: $n = 0/0$: EVEX: $n = 0/0$; EPV-1: $n = 3/18$). AngHV-1, anguillid herpesvirus 1; EPV-1, eel picornavirus 1; EVEX, eel virus European X

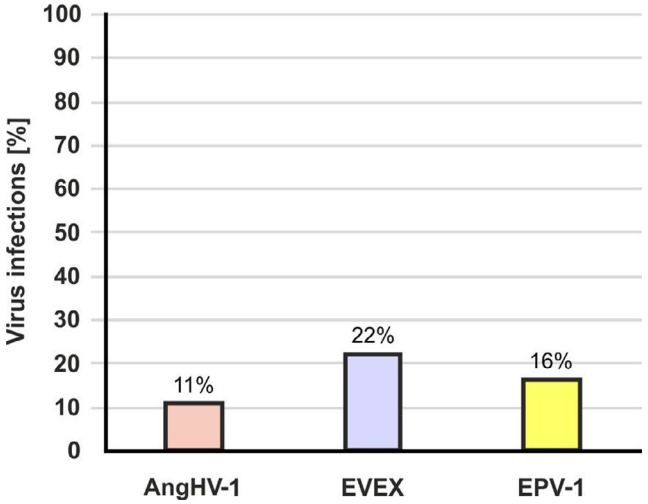


FIGURE 4 Identification of eel pathogens in virus isolates of yellow eels originating from the rivers Rhine, Lippe and Ems via PCR. (AngHV1: $n = 11/100$: EVEX: $n = 22/100$; EPV-1: $n = 16/100$). AngHV-1, anguillid herpesvirus 1; EPV-1, eel picornavirus 1; EVEX, eel virus European X

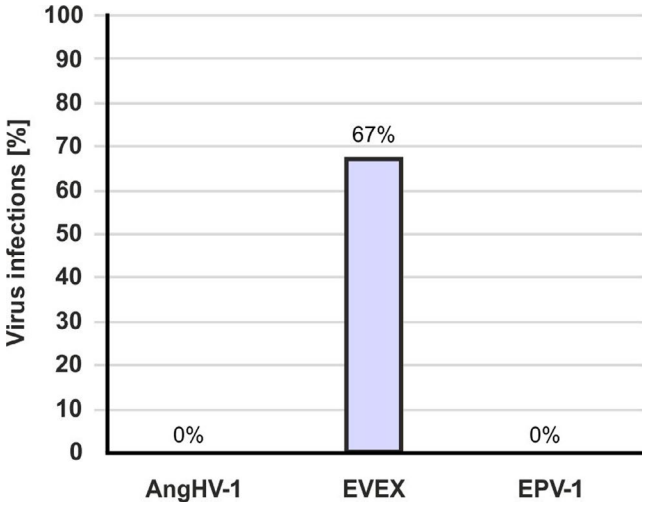


FIGURE 5 Identification of eel pathogens in virus isolates of silver eels ($n = 6$) originating from the river Rhine via PCR. AngHV-1, anguillid herpesvirus 1; EPV-1, eel picornavirus 1; EVEX, eel virus European X

4 | DISCUSSION

The results presented in this study show a wide distribution of AngHV-1, EVEX and EPV-1 in the population of the European Eel in North Rhine Westphalia. Infectious diseases are considered as one of the main causes for the drastic reduction of the European eel panmictic population (Dekker, 2003; Haenen et al., 2009; van Beurden et al., 2012). A factor that has not received much attention in the past years is the reduction of eel health due to infections with pathogenic eel viruses (van Ginneken et al., 2004). Via a combination of cell culture and PCR, we provide first evidence of the circulation of the recently described novel picornavirus EPV-1 in North Rhine Westphalian rivers. In addition, we demonstrate the proof of EPV-1 infections of elvers and yellow eels caught in this location. Yellow eels further carried AngHV-1 and EVEX infections as well as coinfections. Additionally, silver eels were infected with EVEX. To date, the significance of these infections cannot yet be estimated. We found no evidence of disease outbreaks among the animals examined. However, it is assumed that some fish viruses affect blood-forming tissues and might become virulent during stress conditions (Wolf, 1988). Whether a successful migration to the Sargasso Sea of the animals is endangered by the infection status

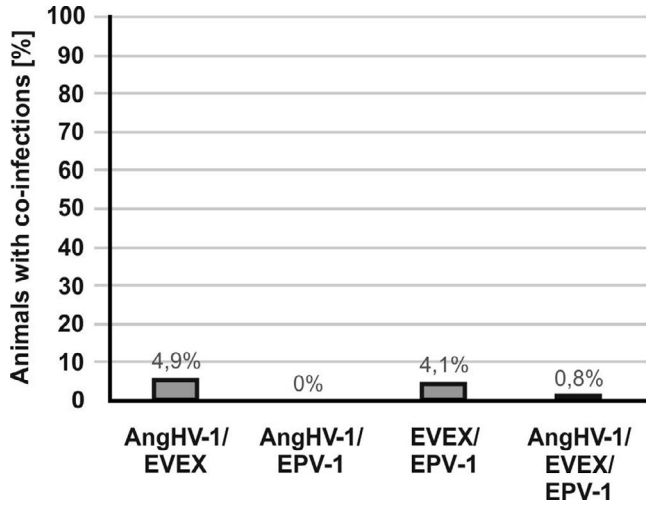


FIGURE 6 Coinfections of the investigated animals ($n = 122$) with the alloherpesvirus anguillid herpesvirus 1 (AngHV-1), the rhabdovirus eel virus European X (EVEX) and the eel picornavirus 1 (EPV-1)

cannot be assessed according to the current state of knowledge. An extensive study based on swim tunnel experiments demonstrated that virus infections severely impede the migration of silver eels (van Ginneken et al., 2005). The impact of EPV-1 infections on the migration has not been investigated yet. Previously, other authors already speculated about picornavirus infections of eels and isolated picornavirus-like particles (Haenen et al., 2009). In 2013, a study provided first proof for a novel picornavirus (EPV-1) isolated from a diseased eel originating from Lake Constance (Fichtner et al., 2013). The authors sequenced the genome of EPV-1 via the Illumina/Solexa method and suggested a novel species in the family Picornaviridae due to its some similarity to conserved P1 and 3CD precursor proteins of other picornaviruses. EPV-1 induced a CPE in EK-1 cells and bath infection experiments revealed a mortality rate of 43% in glass eels which could indicate high impact of the virus on eel populations (Fichtner et al., 2013). However, with the isolation of picorna-like virus also in New Zealand (van Ginneken et al., 2004), the source and the consequences of the distribution of this virus are still unclear and further research is necessary. The initial isolation of EPV-1 from a diseased eel from Lake Constance and isolation of picorna-like virus from European eels from the Netherlands shows that EPV-1 could be present in the whole length of River Rhine.

The river Rhine (and Rhine system) is the main target location for stocking measures in North Rhine Westphalia. In our study, the sampling of this river contained the highest amount of virus-positive eels. The results of the present study should be considered during the planning of future stocking measures. However, further investigations are necessary to accurately estimate the quality of the health status of the present eel population in North Rhine Westphalia. For instance, this could be achieved by an annual virus monitoring with a higher sampling rate.

So far, it was unknown whether EVEX or EPV-1 infections occur in North Rhine Westphalian rivers. EVEX has recently been detected in wild-caught eels in Lough Neagh which suffered from haemorrhagic disease (McConville et al., 2018). Previous studies described the isolation of EVEX from wild and farmed eels and the distribution in different regions in Europe for instance the Netherlands (Haenen et al., 2010) or Italy, Morocco, New Zealand (van Ginneken et al., 2004) and France (Castric & Chastel, 1980; Castric et al., 1984; Jørgensen et al., 1994). Recently, the distribution of virus infections has been extensively studied in the wild eel population in Lough Neagh and EVEX infections have been detected besides infections with AngHV-1 and EVE (McConville et al., 2018). Here, we show the distribution of EVEX as well as EPV-1 in the rivers Ems, Lippe and Rhine and the data might indicate that these infections occur with a higher prevalence compared to AngHV-1. However, this needs to be proven by further annual analyses of animals originating from these rivers. In addition, the investigations should be expanded on other areas. Importantly, the causative agents of 15% ($n = 18/122$) of all virus-positive cell cultures were not identified via the PCR-based techniques which focused on the detection of AngHV-1, EPV-1 and EVEX. Whether other viruses like birnavirus, circovirus, polyomavirus or reovirus caused the CPE was not investigated in this study. The CPE was observed in serial dilutions as well as in additional passages of cell culturing. Further studies are needed to clarify which pathogen caused these unexplained CPEs. A study in Spain (Albufera Lake) demonstrated the circulation of aquabirnavirus and betanodavirus besides AngHV-1 infections (Bandín et al., 2014). Whether these viruses circulate in the European Eel population in Germany need to be investigated.

In conclusion, our study presents new insights into the situation of natural population of eels which are infected with diverse viruses. To date, knowledge on the impact of virus infections relies on a rare number of publications. It needs to be investigated whether larvae or glass eels can already carry eel pathogenic viruses. Especially the distribution of EPV-1 should be studied more closely as there are no data about distribution or impact of this virus. Based on the findings of this study, we speculate that a large number of the present eels in NRW might be impeded in their ability to migrate to the Sargasso Sea, due to highly prevalent viral infections. This knowledge would be fundamental for future eel management plans and stocking measures.

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CONFLICT OF INTERESTS

There are no conflicts of interest to declare for any of the authors.

DATA AVAILABILITY STATEMENT

The data presented in this study are provided in supporting information Appendix S1.

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