Aquaculture International (2020) 28:1811–1823
https://doi.org/10.1007/s10499-020-00559-4

Emergence of viral nervous necrosis is associated with mass mortality in hatchery-reared tilapia (*Oreochromis niloticus*) in Egypt

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Received: 9 March 2020 / Accepted: 5 May 2020 / Published online: 24 May 2020

Abstract
Mass mortalities in Egyptian hatchery-reared Nile tilapia (*Oreochromis niloticus*) fries have negatively impacted the livelihood of fish farmers for the past 3 years due to fish loss (up to 70%), feed waste, and loss of job opportunities. An investigation into possible viral causes of massive die-offs in tilapia during 2018–2019 identified nervous necrosis virus (NNV) strains phylogenetically related to red-spotted grouper NNV. Clinical signs and gross pathology of naturally infected fish were consistent with classical NNV infections. Electron micrographs revealed virions in the retina and brain. NNV RNA was also detected in the brood stocks' brains and ovaries. Experimental infection of NNV-free fries resulted in 100% morbidity and mortality. Since this is the first report of VNN-outbreaks in African tilapia, different aspects of viral epidemiology and possible ways to break the epidemic wave and prevent future interspecies transmission events are discussed.

Keywords *Oreochromis niloticus* · *Betanodavirus* · Mass mortality · Electron microscopy · Hatchery-reared fries · Phylogenetic analysis

Introduction

The increasing demand for high-value inexpensive protein has been driving recent developments in the Egyptian aquaculture sector resulting in a growth that is the strongest among

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10499-020-00559-4) contains supplementary material, which is available to authorized users.

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fisheries-related activities. The growth in Egyptian aquaculture has also created a socioeconomic structure that supports millions of employees (Shaalan et al. 2017). Today, Egypt is ranked ninth worldwide and first in Africa in fish production from aquaculture (FAO 2016). *Oreochromis niloticus* production from Egyptian aquaculture is ranked first nationally and third worldwide, after China and Indonesia (Subasinghe 2015).

Unfortunately, this rapid development of tilapia farming has not been accompanied by the development of an effective health management plan to mitigate the potential risks to this growing industry from introduced or endemic pathogens. The intensification and expansion of tilapia aquaculture have been burdened by outbreaks of mass mortalities in hatcheries and farms allover Egypt. The mortalities were characterized by neurological manifestations, eye opacity, and exophthalmia, in addition to other nonspecific signs (including skin darkening). Efforts were directed to the investigation of possible bacterial, parasitic, and water quality reasons for these massive outbreaks. Many interventions implemented based on the perceived etiology proved of limited value. Little attention was directed to investigating possible viral infections.

Viruses are primary pathogens of aquaculture worldwide. Although members of 12 virus families have been identified in wild and cultured fish species, there is little information about viruses infecting fish in Egypt. Viruses that cause severe disease in tilapia include tilapia lake virus (TiLV) (Nicholson et al. 2017; Surachetpong et al. 2017; Yamkasem et al. 2019), nervous necrosis virus (NNV) (Bigarrø et al. 2009; Keawcharoen et al. 2015), infectious spleen and kidney necrosis virus (ISKNV) (Subramaniam et al. 2016; Suebsing et al. 2016), and lymphocystis disease virus (LCDV) (Paperna 1973).

NNV was of particular interest in the investigation of current mass mortalities in tilapia because viral nervous necrosis (VNN) can cause high mortalities (up to 100%) in larvae and juveniles of many species, it has been reported in almost all continents where aquaculture is practiced, and it causes neurological and ophthalmic manifestations (Shetty et al. 2012) similar to what was reported in the current outbreaks. Additionally, nervous necrosis viruses can maintain an epidemiological cycle in a wide range of temperatures (from 15 to 30 °C) (Binesh and Greeshma 2013).

NNV is a non-enveloped *Betanodavirus* with a reported virion size from 25 to 30 nm. Its genome is positive-sense single-stranded RNA composed of two 5′-capped molecules: a 3.1-kb RNA 1 that encodes the viral RNA-dependent RNA polymerase (RdRp), and a 1.4-kb RNA 2 that encodes the coat protein (CP) (Mori et al. 1992). A sub-genomic RNA 3 is also synthesized during RNA replication from the 3′ terminus of RNA 1. RNA 3 encodes B1 and B2 which are involved in immune evasion (Su et al. 2018).

Four genotypes of betanodaviruses exist: barfin flounder nervous necrosis virus (BFNNV), tiger puffer nervous necrosis virus (TPNNV), striped jack nervous necrosis virus (SJNNV), and red-spotted grouper nervous necrosis (RGNNV) (Nishizawa et al. 1997). Turbot nervous necrosis virus (Johansen et al. 2004) is yet to be formally recognized as a genotype by the ICTV. SJNNV and TPNNV genotypes are known to infect only striped jack and tiger puffer, respectively. RGNNV and BFNNV have a wider host range (Athanassopoulou et al. 2003, 2004; Chi et al. 2003; Hegde et al. 2003; Bigarrø et al. 2009; Bovo et al. 2011; Keawcharoen et al. 2015); RGNNV infect mostly warm-water fishes and BFNNV infect mostly cold-water fishes (Costa and Thompson 2016). Transmission of NNV can be vertical or horizontal (Chi et al. 2001).

Herein, we report the results of a molecular, pathological, and ultrastructural investigation of the viral causes of massive die-offs in tilapia hatcheries during 2018–2019. We also address

the possible mechanisms by which the identified virus was introduced into Egyptian tilapia aquaculture, how long it may have existed in the Egyptian environment, whether it is the primary cause of tilapia mortalities, what can be done to break the epidemic wave, and what actions can be taken to prevent possible future introductions to other compartments of the Egyptian aquatic environment.

Material and methods

Sample collection

Three tilapia hatcheries were sampled during this investigation, two in Kafr El Sheikh (one sampled in July 2018 and the other in July 2019), and one in El Beheira (sampled in July 2019). The stocking densities ranged from 50,000 to 70,000/pond. Fish were fed artificial dry feed for fries containing about 48% protein (Skretting, Egypt). Seventeen alpha methyltestosterone (Argent Laboratories Inc., Philippines) was administered in feed for 2 weeks to produce monosex male tilapia. Twice weekly, 20–30% of water was exchanged. The average ambient temperature during July was 30 °C ± 5 °C. Fries began to exhibit skin darkening, spiraling along their axis, and mortality 5 days after hatching (Online Resource 1). Fries were sampled when mortality was about 50%, however, the mortalities reached 70% by the end of the outbreak. Two hundred clinically affected week-old fries (0.25 ± 0.05 g, 1.5 ± 0.5 cm in length) were sampled per hatchery. Seven brood stocks from El Beheira were collected. Samples were transported directly to the Department of Aquatic Animal Medicine and Management in the Faculty of Veterinary Medicine of Cairo University in an appropriate ice box where they were split and prepared for storage. Samples were then stored at − 80 °C in the Department of Virology until testing. Fish handling was performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Cairo University (Approval number CU/II/F/25/19).

Synthetic positive control preparation

A synthetic positive control standard for five main viral pathogens that threaten aquaculture species was synthesized in silico. The design allowed the amplification of 605 nucleotides of the NNV RNA 2 and 484 nucleotides of segment 9 from TiLV using primers reported elsewhere (Valle et al. 2000; Bacharach et al. 2016) (Table 1). The designed positive control also contained synthetic fragments from lymphocystis disease virus, viral hemorrhagic septicemia virus, and white spot syndrome virus. The synthetic fragment containing viral sequences was cloned in pBlueScript II SK(+)® (Biomatik Corporation, Canada).

Table 1 Primers used for RT-PCR

Primer	Primer sequence	Target	Primer location		Reference sequence
VNNF	5′-ACACTGGAGTTTGAAATTCA-3′	RNA 2	342	361	EF558369.1
VNNR	5′-GTCTTGTTGAAGTTGTCCCA-3′		946	927	
NORTH-S9-F1	5′-TTGCCAGAGCTGAAGCTTATCC-3′	Segment 9	33	54	KU751822.1
NORTH-S9-R1	5′-CGCTTGGTGATGTCACGATGGA-3′		515	494	

Reverse transcription-PCR, sequencing, and phylogeny

Total tissue RNA was extracted from pools of tilapia fries from each hatchery, brood stocks' brains and ovaries, or experimentally infected fish using RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Reverse transcription-PCR (RT-PCR) using Verso 1-Step RT-PCR Kit ReddyMix (ThermoFisher, USA) was used to detect NNV and TiLV in tissue RNA extracts.

RT-PCR was done using Verso 1-Step RT-PCR Kit ReddyMix (ThermoFisher, USA). All components for RT-PCR were mixed in one tube as per manufacturer's instructions, RNA extracts and controls were added to individual tubes and the reaction was allowed to run. Reaction conditions were as follows: a cDNA synthesis step at 50 °C for 15 min; a 2-min incubation step at 95 °C to inactivate the reverse transcriptase; 40 cycles of denaturation at 95 °C for 20 s, annealing at 46.5 °C for VNN or 57.5 °C for TiLV, and extension at 72 °C for 1 min; and a final extension was done for 5 min at 72 °C. For each RT-PCR run, a non-template negative control was prepared by adding an equivalent volume of nuclease-free water instead of the RNA extract. An equivalent volume of the synthetic positive control was used in positive control tubes. The reaction volume was 50 μl. The amplified products were analyzed by electrophoresis on 1.5% agarose gel stained with ethidium bromide.

Amplicons from infected fry pools were sequenced in both directions using a 3500 genetic analyzer (Applied Biosystems™, USA) at Colors Medical Laboratories (Cairo, Egypt). Sequences were compared to reference VNN sequences from different regions of the world using BLAST® of NCBI (Altschul et al. 1990). Nucleotide and deduced amino acid sequence alignments, sequence distances, and identity percentages were done using MegAlign® (DNASTAR 7.0) (Clewley and Arnold 1997) (Online Resource 2). Maximum Composite Likelihood (MCL) and Maximum parsimony phylogenetic trees were calculated from all sequences (Online Resource 2) using MEGA X (Kumar et al. 2018) (Online Resource 3).

Electron microscopy

The whole fry was processed for TEM at the Transmission Electron Microscopy Laboratory, Faculty of Agriculture, Cairo University Research Park. The whole fry was fixed for TEM by 2.5% glutaraldehyde (Sigma Aldrich, St. Louis, Missouri, USA) in phosphate-buffered saline (PBS; 0.1 mol l–1, pH 7.4) for 2 h at 4 °C, post-fixed in 1% osmium tetroxide (Sigma Aldrich, St. Louis, Missouri, USA) in PBS for 1 h at 4 °C, dehydrated in alcohol, and embedded in an epoxy resin. Microtome sections were prepared at approximately 500–1000 μm thickness with a Leica Ultra cut UCT microtome. Semi-thin sections were stained with 1% toluidine blue (Sigma) then sections were examined by HD camera Leica ICC50, Wetzlar, Germany. Ultra-thin sections were prepared further at approximately 30–100 nm thickness and were stained with uranyl acetate and lead citrate. Sections were examined by transmission electron microscope JEOL (JEM-1400 TEM, Peabody, Massachusetts, USA). Images were captured by CCD camera model AMT, optronics camera with a 1632 × 1632-pixel format as side mount configuration at the Transmission Electron Microscopy Laboratory, Faculty of Agriculture, Cairo University Research Park. Zooming and sharpening of images were performed by using image analysis software (Fiji program) (Schindelin et al. 2012).

Experimental challenge

Twenty apparently healthy fingerlings (2–3 g) were collected from a hatchery located away from the regions that reported the clinical syndrome. We depended on two parameters to ensure the health of the fingerlings prior to experimental challenge, first, the fish behavior and appearance after acclimatization for 2 weeks and second, the results of RT-PCR analysis.

The fingerlings were divided equally into two groups. One group was exposed to a homogenate of infected fries containing the Egyptian NT Engy 019 (Online Resource 2) according to the technique described elsewhere (Mori et al. 1991). The control group was not exposed to the infected homogenate. Fish were kept in 2 liters of chlorine-free tap water at ambient temperature (35 ± 2 °C at noon and 25 ± 2 °C at midnight) with continuous aeration. Mortality and clinical signs were observed daily for 1 week. Experimental procedures were approved by Cairo University's Institutional Animal Care and Use Committee (CU- IACUC) (approval number CU/II/F/25/19).

Results

Clinical analysis

Gross external clinical signs led to the suspicion that tilapia fries of this study suffered from VNN disease caused by the NNV. Diseased fries showed skin darkening, lethargy, anorexia, abnormal swimming behavior (swimming in circles, or floating at the water surface near the edges of the pond), eye opacity, and mortalities up to 70%.

Molecular identification, gene sequencing, and phylogenetic analysis

VNN RNA was detected in total RNA extracts of fry pools from the three tested hatcheries, and the extracts of brain and ovaries of brood stocks from El Beheira (605 bp PCR products were generated; Fig. 1). TiLV RNA was not detected in any of the tested extracts.

Partial RNA2 sequences amplified from El Beheira fries, and Kafr El Shiekh fries taken during 2019 and 2018 were deposited in GenBank (accession numbers MN698297, MN698298, and MN701084, respectively). NT Engy 019 showed 99.6% to NT Egypt 019 and 98.3% identity to NT Egypt 018 (Online Resource 4). There were no gaps in any of the sequences when compared to the reference RGNNV (Accession number EF558369). NT Engy 019 was 97.2%–98.9%, 80.2%–82.7%, 76.9%, 75.6%, and 73.2% identical to RGNNV, BFNNV, TPNNV, SJNNV, and TNNV sequences, respectively (Online Resource 4).

The Egyptian NT NNV sequences generally grouped together in phylogenetic analysis and clustered with reference RGNNV sequence (Online Resource 2; Fig. 2). However, NT Egypt 019 and NT Egypt 018 grouped more closely to marine RGNNV isolated from Chinese *Epinephelus akaara* and *Dicentrarchus labrax* NNV isolates from France, Italy, and Tunisia. NT VNN strains previously reported from France and Thailand clustered away from the Egyptian strains reported here and away from each other (Fig. 2); even though the NT France 008 (accession number EU700416.1) was phylogenetically more related to the Egyptian isolates. The clustering pattern described above was also evident in Maximum Parsimony trees (Online Resource 5).

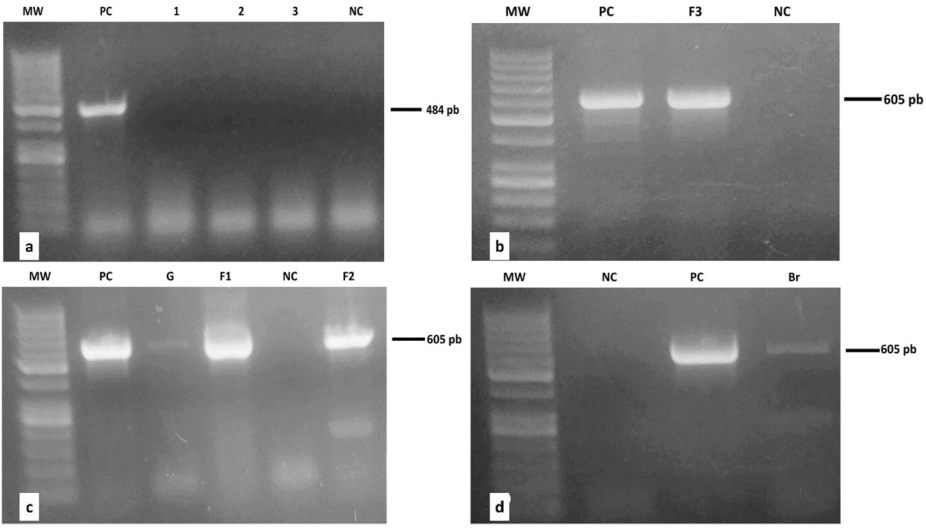

Fig. 1 Detection of NNV nucleic acid in fish tissues. RNA extracted from the whole fry pools from EL Beheira and Kafr El Sheikh. Brain pool and ovary pool of brood stocks from El Beheira were tested for the presence of TiLV and NNV RNA using the developed assay. **a**: TiLV was not detected in tissue RNA extracts from El Beheira sampled fish. 1: Infected fry pool. 2: Ovary pool. 3: Brain pool. **b**: NNV was detected in the tissue RNA extracts from El Beheira sampled fries. F3: Infected fry pool. **c**: NNV was detected in the tissue RNA extracts from El Beheira brood stock samples and Kafr El-Sheikh fry pools. G: Gonads. F1: Fry pool from Kafr El Sheikh 2019. F2: Fry pool from Kafr El Sheikh 2018. **d**: NNV was detected in the brood stocks brain pool RNA extracts from El Beheira. Br: Brain. PC: Positive control. NC: No-template control. MW: GeneRuler® 50 bp DNA Ladder (Thermo Scientific™)

The Egyptian NNV shared T235K amino acid mutation (using GenBank accession # ABU46390.1 numbering). NT Egypt Engy 019 had an additional (D302E) mutation. Moreover, NT Egypt 018 had the unique mutations V253A, L260Q, S278R, G279A, and V272G (compared to other Egyptian viruses). The latter was similar to B Australia 009 (Online Resource 6). NT Egypt Engy 019 amino acid sequence was 100% identical to NT Egypt 019 and 97.2% identical to NT Egypt 018 (Online Resource 7).

Electron microscopy

Electron microscopy of naturally infected tilapia nervous tissue revealed the presence of non-enveloped, icosahedral particles that range in size from 38.2 to 53.5 nm in diameter. Virion density and organization varied according to its proximity to the nuclear membrane and/or what appears to be an inclusion body (Fig. 3). Within the inclusion body evident in Fig. 3b, evenly spaced electron densities formed membrane-bound necklace-like arrangements. The inclusion body also contained an electron dense granule.

Experimental challenge

Tilapia fingerlings exposed to tissue homogenates prepared from naturally infected fries showed the classical signs associated with VNN infection. One fish that died 24 h post-exposure (p.e.) exhibited only mild corneal opacity and skin darkening. The remaining fish died within 7 days p.e. (Online Resource 8). VNN RNA was detected in tissue extracts from

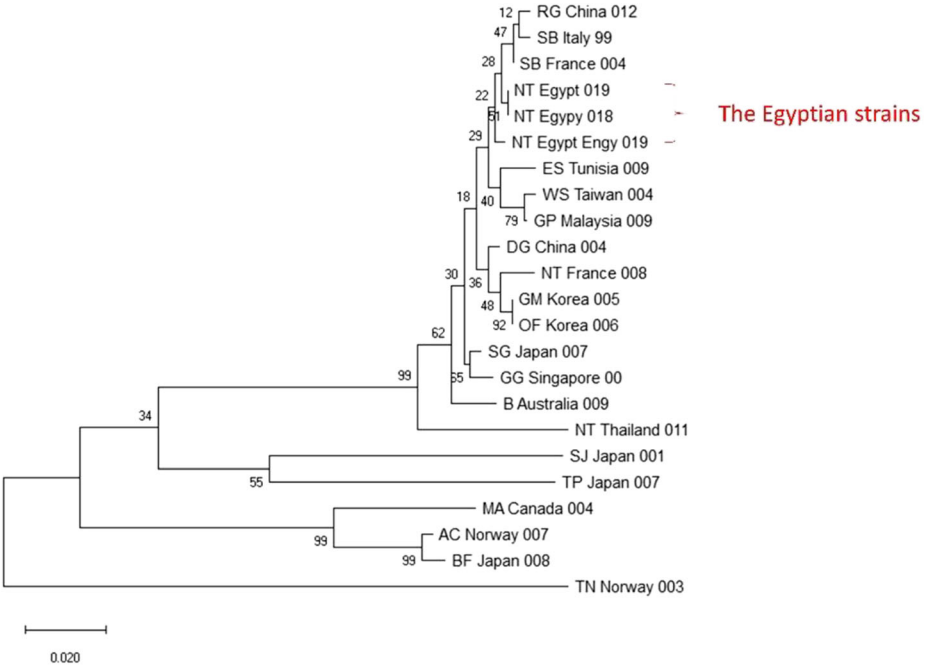

Fig. 2 Phylogenetic relationships between Egyptian NNV sequences and representative sequences from other NNV genotypes. The Maximum Likelihood method and Tamura-Nei model were used for phylogenetic analysis of the Egyptian NNV nucleotide sequences. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 23 nucleotide sequences (Online Resource 2). Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018)

the experimentally infected fish that died after 24 h; viral RNA was not detected in extracts from the one fish that died in the first 24 h p.e (Fig. 4).

Discussion

Mass mortalities (up to 70%) in Egyptian tilapia farms and hatcheries have negatively impacted the livelihood of fish farmers for the past few years. Losses to the national economy due to mass mortalities in aquaculture systems included fish loss and feed waste, as well as job losses; however, the exact value of which is yet to be identified. Water quality and bacterial infections were identified as the main culprits behind the reported tilapia mass mortalities, however, since usual interventions were ineffective in controlling the problem, it was inevitable to investigate other possible causes.

Viruses cause mass mortalities worldwide, however, limited information is available about their impact on the Egyptian aquatic environments. Several reasons may have contributed to this, not least of which is the unavailability of rapid, affordable, and reliable diagnostic tools. Therefore, the development of the synthetic positive control reported here was critical for the investigation of mass mortality causes in cultured tilapia.

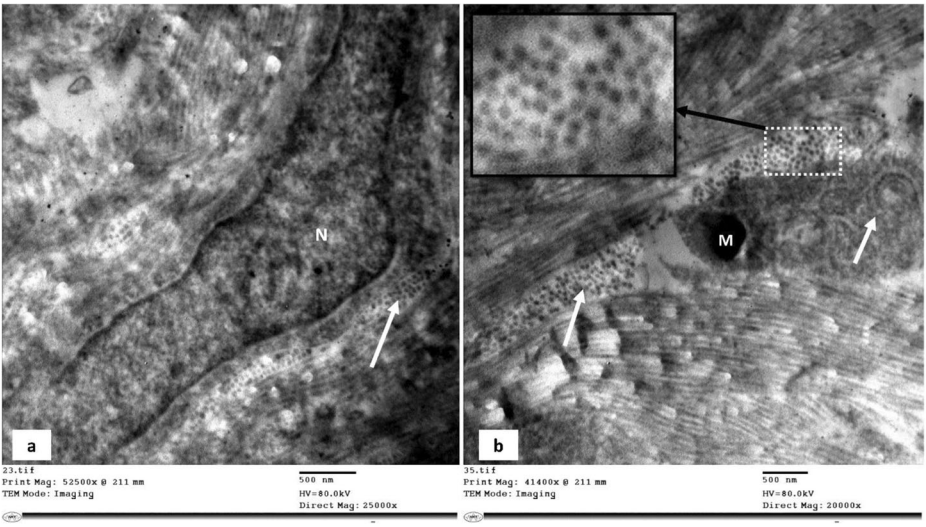

Fig. 3 Electron micrographs of NNV-infected tissue of hatchery-reared tilapia fries. Icosahedral virus particles (38.2–53.5 nm in diameter) were in the cytoplasm of infected cells either in the form of arrays or free in the cytoplasm. **a**: Icosahedral virions arranged in perinuclear arrays of infected cells (N: nucleus). **b**: Virions near to what appears to be an inclusion body. A membrane-bound inclusion body containing an electron-dense melanin-like granule (M) with showing particles in necklace-like arrangements (41,400×, bar = 500 nm)

The design of the synthetic positive control took in consideration the fact that TiLV and VNN can both infect tilapia and share several clinical signs, including nervous manifestations and eye opacity (Bigarrø et al. 2009; Eyngor et al. 2014). In addition, it took into consideration previous reports of the presence of TiLV in Egypt (Nicholson et al. 2017) and VNN in the Mediterranean (Bigarrø et al. 2009). TilV RNA was not detected in any of the extracts tested using RT-PCR.

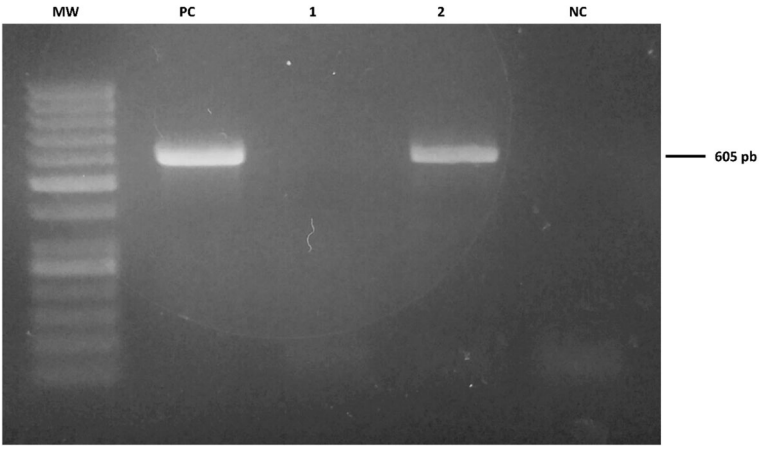

Fig. 4 Molecular identification of viral nucleic acid in experimentally infected fish tissue extracts. VNN RNA was detected in tissue extracts from the experimentally infected fish that died after 24 h; viral RNA was not detected in extracts from the one fish that died in the first 24 h p.e. MW: GeneRuler® 50 bp DNA Ladder (Thermo Scientific™). PC: Positive control. 1: Extract from the single fish that died within the first 24 h p.e. 2: Extract from a fish that died after 24 h p.e. NC: no-template control

On the other hand, VNN RNA was detected in all tilapia tissue extracts tested. This indicated that the hatcheries from which samples were taken had an active outbreak of VNN. Previous reports have indicated that outbreaks of VNN can be a consequence of vertical transmission from infected brood stock (Mushiake et al. 1994). Brood stocks have been reported to become persistently infected (PI). PI brood stocks are difficult to recognize clinically but are a primary source of environmental contamination with VNN (Johansen et al. 2002). The stress of repeated spawning has been suggested to increase VNN vertical transmission frequency (Mushiake et al. 1994). Since not all infected offspring die during an outbreak, it is projected that a certain percentage of the surviving population will also become PI fish that continue the lifecycle on the facility or become a source of infection for other aquaculture facilities if they were sold. A state of persistent infection has been reported in juvenile Atlantic halibut (*Hippoglossus hippoglossus*) that appeared normal but carried VNN in the brain, retina, and internal organs, especially associated with macrophage-like cells (Johansen et al. 2002).

Now that infection of Egyptian tilapia with NNV has been established, the following questions became pertinent: First, what is the genetic relationship between the Egyptian virus and known VNN genotypes? Second, how did the Egyptian Nile tilapia get the virus? Third, how long has the virus been in the Egyptian environment? Fourth, is NNV the primary cause of tilapia mortalities? Fifth, what can be done to break the epidemic wave? Finally, how can we prevent possible future interspecies transmission events?

Phylogenetic analysis revealed that the Egyptian *Oreochromis niloticus* NNV is more genetically related to the RGNNV than any of the other three established genotypes of *Betanodavirus* (Nishizawa et al. 1997), or the proposed turbot NNV genotype (Johansen et al. 2004). The three Egyptian VNN sequences reported here clustered with Mediterranean NNV isolates from French, Italian, and Tunisian seabass (*Dicentrarchus labrax*) (Skliris et al. 2001; Thiéry et al. 2004; Chérif et al. 2010). This clustering suggests that one of these Mediterranean viruses is the potential origin of one or more of the Egyptian NNV, however, it does not explain how the viruses reached Egyptian tilapia farms. For this, it is important to consider NNV ecology and epidemiology as well as aquaculture practices of Egyptian farmers.

VNN is known as a principal problem in Mediterranean wild and farmed marine fish species (OIE 2017), including European sea bass *Dicentrarchus labrax* (*L.*) reared in freshwater in Italy and Greece (Athanassopoulou et al. 2003; Bovo et al. 2011). In Egypt, there is another bridge linking Mediterranean marine fishes and tilapia aquacultures. Most tilapia aquaculture systems are polyculture, rearing tilapia and mullet in the same pond. *Mugilidae* species are migratory euryhaline fish that can withstand a wide range of salinity levels and are reared with tilapia for economic reasons. Mullet fries are captured in collection stations on the Mediterranean Delta coast at the estuaries of the River Nile branches and the major agricultural drainage canals, and the canal connecting lagoons and lakes to the sea (Saleh 2008). *Mugilidae* species are susceptible to VNN infection (Ciulli et al. 2007) and the virus has been reported in wild mullet of the Mediterranean basin (Ucko et al. 2004). Mullet can act as an apparently normal carrier of NNV (Gomez et al. 2004) and NNV can remain infectious in a wide range of temperatures (Binesh and Greeshma 2013). Therefore, it is fathomable that coculture of both species in the same pond under a certain set of environmental conditions will allow for the selection from the quasispecies population in a manner that favors adaptation to Nile tilapia.

Adaptation to a host involves efficient receptor recognition, evasion of host immune mechanisms, and compatibility between viral and cellular replication machinery (Costa and Thompson 2016). Analysis of the predicted amino acid sequence of the sequenced PCR

products revealed the presence of mutations within the protruding P domain of the viral capsid protein associated with host receptor binding and cell specificity (Chen et al. 2015). The one unique T235K mutation of the Egyptian VNN strains' capsid protein does not allow us to make substantiated conclusions regarding its influence on efficient entry to Nile tilapia cells. The other five unique mutations of the 2018 Egyptian NNV (compared to the 2019 Egyptian NNV) may be an indication that it has existed in the Egyptian Nile tilapia for a longer time, and it is probably the parent of the Egyptian viruses currently in circulation. RNA viruses with positive single-stranded genomes are known to adopt divergence mechanisms lacking a "clock-like" behavior to evolve distinct phenotypes of capsid proteins to allow rapid adaptation to new fish species and environments (Toffolo et al. 2007).

An alternative scenario wherein NNV may have been present in the River Nile ecosystem prior to the observed outbreaks in tilapia requires the existence of a trigger(s) for VNN induction that was(were) not present before, quantitatively or qualitatively. Intensification and bad water quality are known predisposing factors for eruption of diseases (Shetty et al. 2012). However, available data are not enough to make conclusions regarding this assumption. An investigation into the existence of carriers in hatcheries and wild fish upstream of the reported outbreaks or at the Aswan Dam may provide the necessary evidence to support such a hypothesis.

The magnitude of the outbreak in Egypt, i.e., the widespread mortalities in aquaculture facilities in the different governorates of the Nile Delta and along the Mediterranean, is also related to virus characteristics and aquaculture practices. Infected water may act as an abiotic vector because of the high resistance of the virus to relatively high temperatures (up to 37 °C) and acidic conditions (OIE 2017). Thus, fresh water can carry the virus along water flow routes during outbreaks in tilapia farms, and marine water can carry the virus to freshwater populations during acclimatization of the captured mullet. The virus may also spread from one hatchery to another during clinical outbreaks on personnel and/or fomites.

Koch's postulates were used to address the question regarding whether NNV was the primary cause of the observed pathology in Nile tilapia. Because the disease was replicated in experimentally infected tilapia fries under controlled conditions, it was concluded that outbreaks in the sampled populations were indeed caused by NNV infection. This high mortality observed experimentally may be attributed to the high concentration of infectious viruses in the confinement of the disease tank. This phenomenon was observed during experimental infection of striped jack larvae as well (Arimoto et al. 1993). The water temperature during experimental infection may have also played a role in the observed mortalities during experimental infection. High temperature was shown to accelerate reaching maximum (100%) mortalities of grouper larvae challenged with GNNV; from 50 h post-challenge (h.p.c.) when conducted at 28 °C to 80 h.p.c. at 24–28 °C (Chi et al. 1999).

It is also important to report that the average size of the Egyptian NNV particles in naturally infected tilapia fries was larger than that previously reported (Sahul Hameed et al. 2019). This may be an indication that there are mutations elsewhere in the genome that influence capsomere number and/or 3D structure. Electron micrographs of infected tissue otherwise presented the picture reported elsewhere (Johansen et al. 2002), with capsid structures visible in membrane-bound inclusion bodies containing melanin-like granules; melanin-like granules may be a sign of infection of melanomachrophages that had migrated to the site of infection as a part of a failed defense mechanism.

For now, it is important to overcome the crest of the outbreak as soon as possible to stop the loss to the economy and subsequent socioeconomic impact. It is recommended to upgrade

biosecurity practices in hatcheries to prevent spread to farms. First, the developed diagnostic assay should be used to screen all brood stocks. Only NNV-free hatcheries should be certified to sell fries to commercial farmers. The developed assay should also be used to screen other fish species intended for freshwater or marine aquaculture, and fish movement should be linked to freedom from NNV infection, even if apparently normal. A quantitative upgrade of the developed diagnostic assay will also help research investigating the feasibility of solutions for disinfection of water intended for hatcheries, and if possible, to other farms.

Finally, there is a real possibility that a reverse jump of VNN from *Oreochromis niloticus* to marine cultured fishes can happen due the practice of using living undersized tilapia to feed carnivorous fishes like meager (*Argyrosomus regius*). Therefore, screening of fish intended for carnivorous fish feeding is also recommended. Alternatively, although less feasible, artificial feed could be used until the current VNN outbreak has been controlled.

In conclusion, this research documents that the cause of recent unexplained massive die-offs in Egyptian Nile tilapia are NNV strains phylogenetically related to RGNNV from Mediterranean seabass. It also provides evidence that the Egyptian NNV strains have been present for several years in the Nile ecosystem, and it provides a reasonable explanation for how NNV may have been introduced to Egyptian tilapia aquacultures. Finally, possible intervention strategies are suggested to help control the current outbreak and prevent possible future reverse jumps into cultured marine fish species.

Availability of data and material The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Engy Taha, Mohamed Shawky, Basem Ahmed, Mohamed Moustafa, Ausama Yousif, and Mohamed Abdelaziz. The first draft of the manuscript was written by Engy Taha and Ausama Yousif and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding This research has been partially funded by the Faculty of Veterinary Medicine of Cairo University's Graduate Research Support Fund.

Compliance with ethical standards

Fish handling was performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Cairo University (Approval number CU/II/F/25/19).

Conflict of interest The authors declare that they have no conflict of interest.

Consent to participate All authors consented to participate in all aspects of this research and publication.

Consent for publication All authors agree to the publication of this manuscript in Aquaculture International.

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