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Betanodavirus infection in reared marine fishes along the Arabian Gulf

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Abstract *Betanodavirus* or nervous necrosis virus (NNV) is responsible for viral nervous necrosis (VNN) of fishes and is associated with mass mortalities of various fish species throughout the world. Here, we report for the first time *Betanodavirus* infection in Arabian Gulf region of the Kingdom of Bahrain. The present study is also the first report of mortality due to NNV infection in sobaity seabream *Sparidentex hasta*. NNV was detected in reared fry of gilthead seabream *Sparus aurata* and adults of sobaity seabream and brown-spotted grouper *Epinephelus chlorostigma* using nested PCR. In the present study, the rearing unit recorded 100% mortality in fry of gilthead seabream and 24 and 63% mortalities were recorded in different age groups of adult sobaity seabream due to VNN. Classical clinical signs of VNN such as body discoloration, loss of appetite and abnormal swimming behaviour were observed in fry of gilthead seabream. Histopathological studies revealed characteristic vacuolation and degeneration of brain and ocular tissues of sobaity seabream. However, brown-spotted grouper carried subclinical NNV infection, and there was no mortality in the group. Sequencing of nested PCR products and real-time PCR further confirmed the presence of the virus.

Keywords *Betanodavirus* · VNN · Seabream · Grouper · Arabian Gulf

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Introduction

The Arabian Gulf consists of coastline of eight countries which include Bahrain, Iran, Iraq, Kuwait, Oman, Qatar, Saudi Arabia and United Arab Emirates which accounts for a total length of approximately 989 km^2. Aquaculture development started late in the region and still in infancy. Commercial mariculture in the Kingdom of Bahrain started in 2014. Presently, several marine and fresh water fish and shellfish species are cultured successfully. The National Mariculture Centre (NaMaC) situated in the Kingdom of Bahrain has been successful in achieving mass seed production of some commercially important marine fin fish species such as rabbit fish (*Siganus canaliculatus*), sobaity seabream (*Sparidentex hasta*), gilthead seabream (*Sparus aurata*), mangrove red snapper (*Lutjanus argentimaculatus*) and brown-spotted grouper (*Epinephelus chlorostigma*). Development of aquaculture in the region has paved the way for several bacterial and viral infections in commercially reared fishes and shell fishes. Among various viruses infecting fish, *Betanodavirus* or nervous necrosis virus (NNV) infection is common and has been reported infecting nearly 70 species of marine and fresh water fishes covering different geographical region (Costa and Thompson 2016; Doan et al. 2016; Shetty et al. 2012). NNV belongs to the family *Nodaviridae*, and recent studies have classified this family of viruses in to three different genera viz., *Alphanodavirus* infecting insects, *Betanodavirus* infecting fishes and the third new group is *Gammanodavirus* infecting shrimps and fresh water prawns (Doan et al. 2016; NaveenKumar et al. 2013; Nishizawa et al. 1997). Disease caused by *Betanodavirus* is commonly called viral nervous necrosis (VNN) or sometimes, it is also called as viral encephalopathy and retinopathy (VER) as the virus damages the central nervous system and eyes of the infected fish. VNN/VER is histopathologically characterized by vacuolation of brain, spinal cord and retina (Azad et al. 2005; Hegde et al. 2002; Keawcharoen et al. 2015).

VNN is one among the many devastating diseases which can cause mortalities up to 100% (ranging from 15 to 100%), particularly in earlier stages of the fish life cycle (larvae, fry, fingerlings) (Azad et al. 2005; Munday et al. 2002; Shetty et al. 2012). Reports related to mortalities in older and market-size fishes due to VNN are also available (Fukuda et al. 1996; Kara et al. 2014). Groupers, seabass and flatfishes are generally considered to be more susceptible to infection with NNV (OIE 2015; Sano et al. 2011), whereas seabream such as *S. aurata* is considered to be resistant and are reported to be an asymptomatic carrier host (Aranguren et al. 2002; Castric et al. 2001). Clinical signs involve lack of appetite, changes in pigmentation in some cases, swim bladder hyperinflation, floating belly-up due to inflated swim bladder and erratic swimming behaviour of fishes (Azad et al. 2005; Johansen et al. 2004; Munday et al. 2002; Shetty et al. 2012). Several studies have shown that NNV can be both vertically and horizontally transferable (Azad et al. 2006; Breuil et al. 2002; Munday et al. 2002; Shetty et al. 2012).

Betanodavirus similar to other nodaviruses are non-enveloped viruses consisting of two single-stranded, positive sense RNA (RNA1 and RNA2) as their genetic material, and the size of the viruses range from 25 to 32 nm. RNA1 is 3.1 kb in size and encodes genes for RNA-dependent RNA polymerase enzyme (110 kDa) and B2 protein (14.7 kDa), and RNA2 which is of 1.4 kb in size encodes for the capsid protein (Fenner et al. 2006; Iwamoto et al. 2001, 2004; Mori et al. 1992).

NNV infection is difficult to control, and there are no commercially available vaccines for VNN. Hence, it is very important to have proper management tools in place during aquaculture production, and early diagnostics is one such tool which can reduce the impact of VNN.

Several methods including molecular and serological methods are available for the detection of NNV among which reverse transcriptase polymerase chain reaction (RT-PCR) is the most commonly used method (Azad et al. 2005; Keawcharoen et al. 2015). However, nested RT-PCR has been found to be more sensitive than the regular RT-PCR methods (Thiery et al. 1999; Dalla Valle et al. 2000). Generally, real-time PCR is considered more sensitive than the conventional PCR assays. Both SYBR green- and TaqMan®-based real-time PCR assays have been standardized and validated for the detection of VNN (Baud et al. 2015; Dalla Valle et al. 2005; Hick and Hittington 2010; Panzarin et al. 2010). Applied Biosystems™ has developed a TaqMan® probe-based assay (VetMAX™-Plus qPCR Master Mix based) for the detection of VNN.

VNN has been officially reported from several countries. However, there are no published reports of VNN in the Arabian Gulf region of the Kingdom of Bahrain. The present study is the first report of VNN and NNV infection from the Kingdom of Bahrain and also the first report on NNV infection and VNN inflicted mortality in sobaity seabream.

Materials and methods

Sample collection

Samples were regularly collected from various marine fish hatcheries, nurseries and culture systems along the Arabian Gulf for the surveillance of important viral pathogens. Samples of the present study were collected from marine fish nursery and rearing unit located in NaMaC, Kingdom of Bahrain, which saw sudden mortalities of gilthead seabream (*S. aurata*) fry and adults of sobaity seabream (*S. hasta*). Abnormal swimming behaviour, loss of appetite and body discolouration indicated VNN. The unit consisted of gilthead seabream fry weighing about 2 g, two groups of sobaity seabream (first group consisted of fishes weighing around 1 kg and second group had fishes weighing around 2 kg) and brown-spotted groupers (*E. chlorostigma*) (weighed more than 4 kg) maintained separately in individual tanks. Fry of gilthead seabream were imported from Greece, and sobaity seabream and groupers were raised from wild caught fish fry from local waters. Fry and adults were maintained in a flow-through system consisting cement tanks containing sea water with continuous aeration and water exchange was done daily. Fry were fed twice daily with commercially available feed at the rate of 5% of the body weight, and the adults were fed with trash fishes. Temperature variation ranged from 26 to 30 °C, and the salinity variation was between 35 and 38 ppt.

Dead/moribund fish showing clinical signs of VNN were collected in the case of both gilthead seabream and sobaity seabream. Samples from brown-spotted grouper showing no clinical signs were also collected suspecting horizontal virus transfer. In case of fry, whole head of the fish was used and in case of adults, target organs (brain and eye) were collected for further processes. Live fishes were sacrificed by overdosing with MS-222 (Sigma-Aldrich, USA) to remove target organs. Total of five fishes were sampled in each case. Samples were washed in DEPC-treated water, transferred to RNase-free sterile tubes containing RNAlater (25 mM sodium citrate, 10 mM EDTA, 70 g ammonium sulphate/100 ml of solution, pH 5.2) and transported to laboratory using ice-chilled box. Upon arrival, RNAlater drained samples were stored at −80 °C until RNA extraction which was carried out after thorough maceration. For histology, samples were fixed in 10% buffered neutral formalin (100 ml formalin, 900 ml water, 4 g/l NaH_2PO_4 and 6.5 g/l Na_2HPO_4). Samples from kidney and liver were taken on

tryptic soya agar plates which were incubated at 30 °C for 48 h to check for bacterial infection. Sampling and testing, as described above, were carried out twice for confirmation.

Histopathology

Sections were taken from the fixed tissue, washed in tap water and passed in serial dilutions of alcohol for dehydration. Specimens were cleared in xylene and embedded in paraffin. Paraffin wax tissue blocks were sectioned (5–7 μm thickness) using microtome (microTec® CUT, Germany). The obtained tissue sections were collected on glass slides, deparaffinized and stained by haematoxylin and eosin (Bancroft et al. 1996). Prepared slides were examined for histopathological lesions using a light microscope (Olympus BX50, USA).

Total RNA extraction

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions with some modification and stored at −80 °C until used. For every 250 μg of the tissue, 750 μl of TRIzol reagent was added and homogenized, followed by incubation at room temperature (28 ± 1 °C), for 10 min. Following incubation, 200 μl of chloroform was added to the sample, mixed vigorously and centrifuged at 12,000*g* for 10 min at 4 °C. The aqueous phase obtained was transferred to a fresh tube, to which 500 μl of isopropyl alcohol was added and incubated at room temperature for 20 min for precipitating out RNA. The sample was centrifuged at 12,000*g* for 15 min at 4 °C to obtain RNA pellet. RNA pellet obtained was washed with 75% ethanol and then dissolved in 50 μl DEPC-treated ultrapure water (Elga, UK). The concentration and purity of RNA was determined using NanoDrop™ 2000 spectrophotometer (Thermo Scientific, USA).

Nested RT-PCR

Complimentary DNA was reverse transcribed from total RNA (~1 μg) using 2 μl region specific (VNNV2, Table 1) reverse primer (100 ng/μl) and GeneAmp® RNA PCR Core Kit (Thermo Scientific, USA) at 37 °C for 30 min. After reverse-transcribing, PCR was carried out in a 30 μl reaction mixture containing 1× assay buffer with 1.25 mM $MgCl_2$, 200 μM of each of the 4 dNTPs, 1 U of Taq polymerase and 10 pmol of each primer. PCR was performed in a thermal cycler (BioRad, Singapore) with initial denaturation of 95 °C for 5 min and 30 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 45 s with a final delay of 5 min at 72 °C. The amplified PCR products were resolved by electrophoresis on 2% agarose gel, stained with ethidium bromide (0.5 μg/ml) and photographed using a gel documentation system (BioRad, USA). OIE (2015) recommended primer sets VNNV1 and VNNV2, and VNNV3 and VNNV4 were used for first and second step PCR (nested PCR reaction), respectively (Table 1). VNNV3 and VNNV4 primer set targets region within the first step PCR product. Nested PCR was performed using 1 μl of PCR product from first step PCR. PCR reaction mixture (except primers) and conditions for second step were the same as the first step PCR. Both the PCR products were sequenced using both forward and reverse primers with an automated ABI 3100 Genetic analyser using florescent label dye terminators (SciGenom, Cochin, India).

Table 1 Sequences and amplicon size of the primers used in the present study

Sequence (5′-3′)	Primer	Target	Amplicon size (bp)	Reference
ACACTGGAGTTTGAAATTCA	VNNV1	RNA2	605	OIE 2015
GTCTTGTTGAAGTTGTCCCA	VNNV2			
ATTGTGCCCCGCAAACAC	VNNV3		254	
GACACGTTGACCACATCAGT	VNNV4			

Real-time PCR

Complementary DNA (cDNA) synthesis (reverse transcription) from total RNA (~1 μg) was done using random hexamers (final concentration of 2.5 μM) using GeneAmp® RNA PCR Core Kit (Thermo Scientific, USA) at 37 °C for 30 min. Real-Time reaction was carried out in StepOnePlus™ Real-Time PCR system (Applied Biosystem™, USA) using 2× qPCR VetMAX™-Plus qPCR master mix (Applied Biosystems™, USA) and Virus Nervous Necrosis TaqMan™ assay kit (Applied Biosystems™, USA) following the manufacturer's instructions. Primer (400 nM each forward and reverse) and probe (120 nM) mixture was used with 8 μl of 2× qPCR VetMAX™-Plus master mix to a total volume of 20 μl reaction. Two microliters of cDNA was used. Real-Time PCR instrument was programmed using recommended thermal-cycling conditions for MGB probe with FAM reporter and non-fluorescent quencher (Virus Nervous Necrosis TaqMan™ assay kit). Enzyme activation was done at 95 °C for 10 min and 40 cycles of amplification at 95 °C for 15 s and 55 °C for 45 s was carried out. To minimize subjectivity, threshold and baseline were kept in auto mode. Suitable negative control (nuclease-free water) and positive control (in-house) were used for quality control.

Results

Clinical examinations

During investigation, 100% mortality was observed in fry of gilthead seabream. Tank consisting 1 kg sobaity seabream showed mortality of 63% and tank with 2 kg sobaity seabream showed 24% mortality, 55 and 12 out of 88 and 50, respectively. No mortality was seen in brown-spotted grouper. However, subsequent PCR analysis showed brown-spotted grouper to be positive for NNV. All mortalities occurred within 3 weeks of first appearance of clinical signs. Fry of gilthead seabream and adult sobaity seabream showed clinical signs of NNV infection, while brown-spotted grouper remained asymptomatic. Clinically, diseased gilthead seabreams were lethargic and showed abnormal swimming behaviour (vertical positioning, spinning and flexing of body), loss of appetite and body discolouration. On the other hand, haemorrhages were observed on abdomen, operculum and caudal and pelvic fins of diseased adult sobaity seabream. Some adult sobaity seabream were observed floating on the surface with belly-up position. Sixty five percent infected sobaity seabream showed hyperinflation of swim bladder. Darkening of the whole body in infected sobaity seabream was also observed in 20% fishes. Samples taken from kidney and liver did not show any bacterial growth on tryptic soya agar plates.

Histopathology (light microscopy)

The histopathological examination of NNV-infected sobaity seabream revealed severe pathological lesions in the brain (Figs. 1 and 2) and ocular tissues (Fig. 3). In the brain, oedema of primitive meningeal layer, severe congestion of the blood vessels with haemorrhages in submeningeal spaces, was observed. Mononuclear inflammatory cell infiltration was not uncommon. The main type of inflammatory cells in affected parts was lymphocytes; the cells arranged as a perivascular cuff around the blood vessels of the brain tissue. Severe congestion of the cerebral blood vessels as well as melanophore aggregation was also noticed in the brain tissue in some examined cases. Characteristic intracytoplasmic inclusion bodies were noticed in some of the glia cells in the brain tissue. The inclusions appeared as large, rounded bodies pushing the nuclei of the infected cells to one side of the cytoplasm. Chromatolysis was also noticed in the Purkinje cells where the Nissl's granules were arranged in the periphery of the cytoplasm. In some areas of the brain tissue of infected fishes, neuronal degeneration, gliosis and neuronophagia were noticed. Multiple vacuolated areas (status spongiosus) were common in most of the examined cases. Intracellular oedema of the brain tissue was also observed in some cases.

In ocular tissue (Fig. 3), the lesions were observed mainly in retina, optic nerve and periorbital tissues. In retina, marked vacuolation of both inner and outer granular layer of the retina was a common finding. Haemorrhage in sub-epithelial region of pigmented epithelium was also noticed. In optic nerve, oedema was noticed while the periorbital tissue showed congestion of the blood vessels, mononuclear cell infiltration as well as melanophore aggregation.

Molecular detection

All suspected samples, fry of gilthead seabream and adults of sobaity seabream and brown-spotted grouper were positive for NNV with a product size of 605 bp for the first step PCR and 254 bp for the second step PCR as expected (Fig. 4). PCR products were sequenced for confirmation of the NNV sequence. Sequence of both 605 and 254 bp size amplicon showed 100% similarity with previously published NNV sequences. For further confirmation a real-time PCR assay that was optimized for the detection of *Betanodavirus* by Applied Biosystem™, USA, was also used. Real-time PCR assay showed the suspected samples to be positive for NNV with a threshold cycle (C_T) value ranging from 17 to 22 which was comparatively lower with our earlier real-time assays. For more confirmation, sampling and detection assays were carried out twice and the results remained unaltered.

Discussion

Betanodavirus infection has been reported across the globe among different fish species and is considered to be a serious threat for the growth of aquaculture industry (Costa and Thompson 2016; Doan et al. 2016; Shetty et al. 2012). Mortalities due to *Betanodavirus* in both young as well as older fishes have been reported (Azad et al. 2005; Fukuda et al. 1996; Kara et al. 2014). The present study is the first report of mortalities due to *Betanodavirus* infection from the Arabian Gulf coast of the Kingdom of Bahrain. We report the detection of *Betanodavirus* in fry of gilthead seabream (*S. aurata*) and adult brown-spotted grouper (*E. chlorostigma*). We also report for the first time, mortalities due to NNV in sobaity seabream (*S. hasta*). Hundred

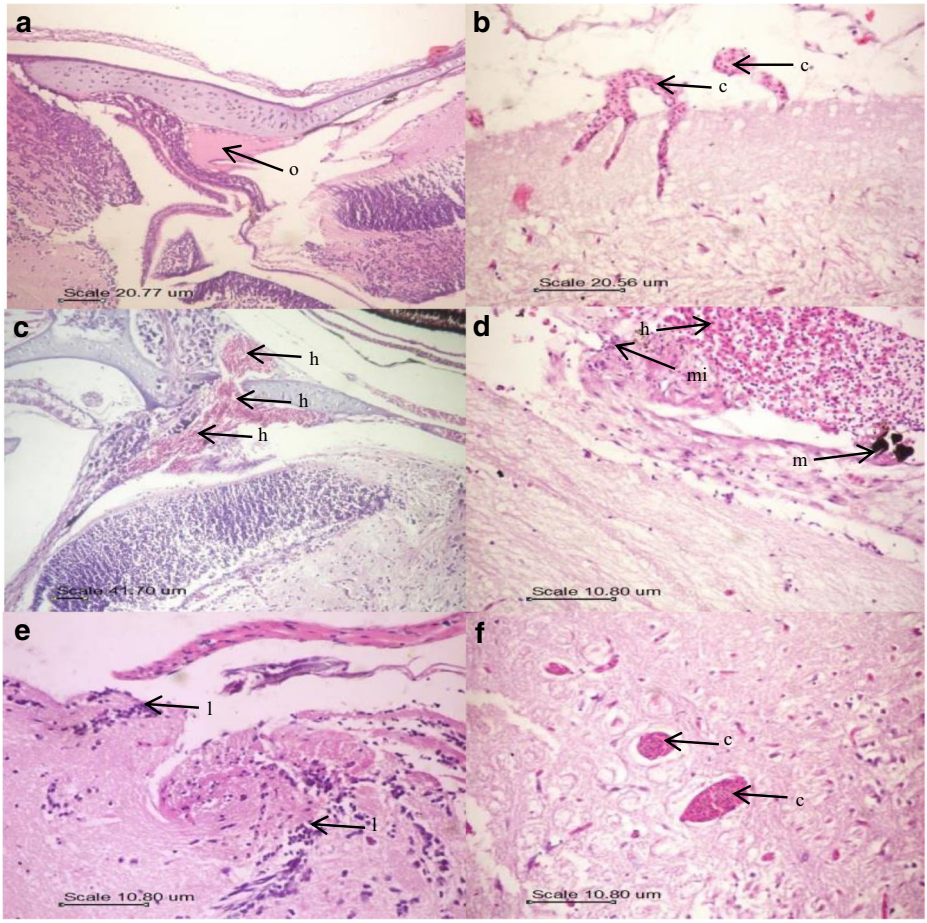

Fig. 1 Histopathological examination (H&E stain) of brain of the diseased sobaity seabream *Sparidentex hasta* showing **a** oedema (*o*) in the meningeal region. **b** Severe congestion (*c*) of meningeal blood vessels. **c** Haemorrhage (*h*) in subarachnoid. **d** Submeningeal haemorrhage (*h*), mononuclear cells infiltration (*mi*) and melanophores aggregation (*m*). **e** Perivascular cuffing of lymphocytes (*L*). **f** Severe congestion (*c*) of cerebral blood vessels

percent mortality was recorded in fry of gilthead seabream and considerable mortality was also observed in the adults of sobaity seabream. Brown-spotted groupers were also found to be positive for NNV; however, no mortality was observed.

Initially, sudden mortality was observed in the fry of gilthead seabream. When observed for clinical signs, classical signs of *Betanodavirus* infection which included abnormal swimming behaviour, loss of appetite and body discolouration similar to the earlier reports (Azad et al. 2005; Johansen et al. 2004) were seen. Some earlier reports suggest gilthead seabream to be resistant to VNN and act as healthy carriers of NNV (Castric et al. 2001). There are also reports of experimental *nodavirus* transmission showing mortalities of not more than 50% in gilthead seabream fry weighing 2 and 4 g (Aranguren et al. 2002). Bitchava et al. (2007) observed 19% mortalities due to VNN in gilthead seabream. However, in the present case study, we observed 100% mortality of gilthead seabream (2 g fry) due to VNN. Difference in the pathogenicity of the genetic variant of NNV may be one of the reasons for highest level of mortality observed.

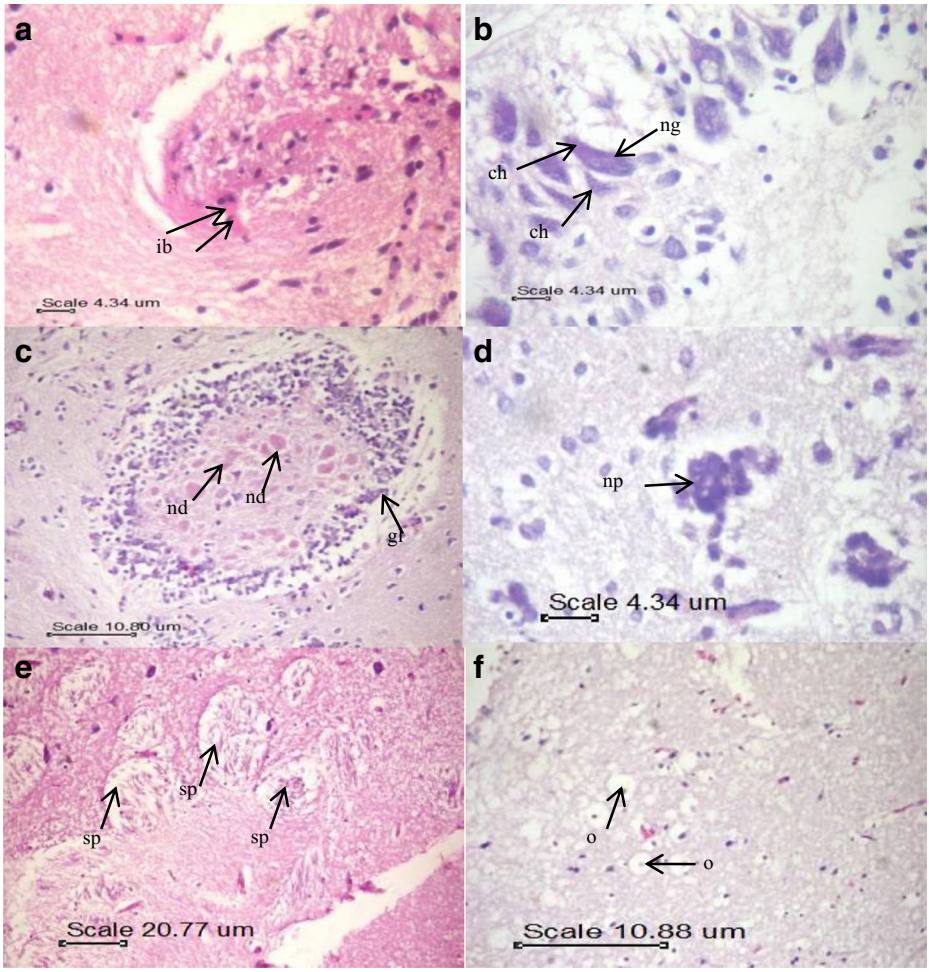

Fig. 2 Histopathological examination (H&E stain) of brain tissue of diseased sobaity seabream *Sparidentex hasta* showing **a** eosinophilic intracytoplasmic inclusion bodies (*ib*). **b** Chromatolysis (*ch*) and arrangement of nessel's granules (*ng*) in the cytoplasm. **c** Neuronal degeneration (*nd*) and gliosis (*gl*). **d** Neuronophagia (*np*). **e** Status spongiosus (*sp*). **f** Cerebral oedema (*O*)

Subsequently, mortalities were also observed in adult sobaity seabream. Affected adults of sobaity seabream showed haemorrhages on the body. Hyperinflation of swim bladder which is common in VNN (Kara et al. 2014; Vendramin et al. 2013) was also observed. Haemorrhages were also evident on the internal organs. This is the first report of NNV infection and mortalities due to VNN in sobaity seabream. Histopathological examinations were carried out for NNV-infected sobaity seabream. Several earlier studies have indicated that brain, retina and spinal cord are the target organs of NNV (Bitchava et al. 2007; Keawcharoen et al. 2015). Vacuolation, degeneration and necrosis of spinal, ocular and brain tissues are characteristics of VNN (Hegde et al. 2002). Our study also showed similar results, and our findings revealed that the main pathological lesions in case of VNN were mainly noticed in the brain and ocular tissue. In the present study, histology of nodavirus-infected tissue also revealed marked

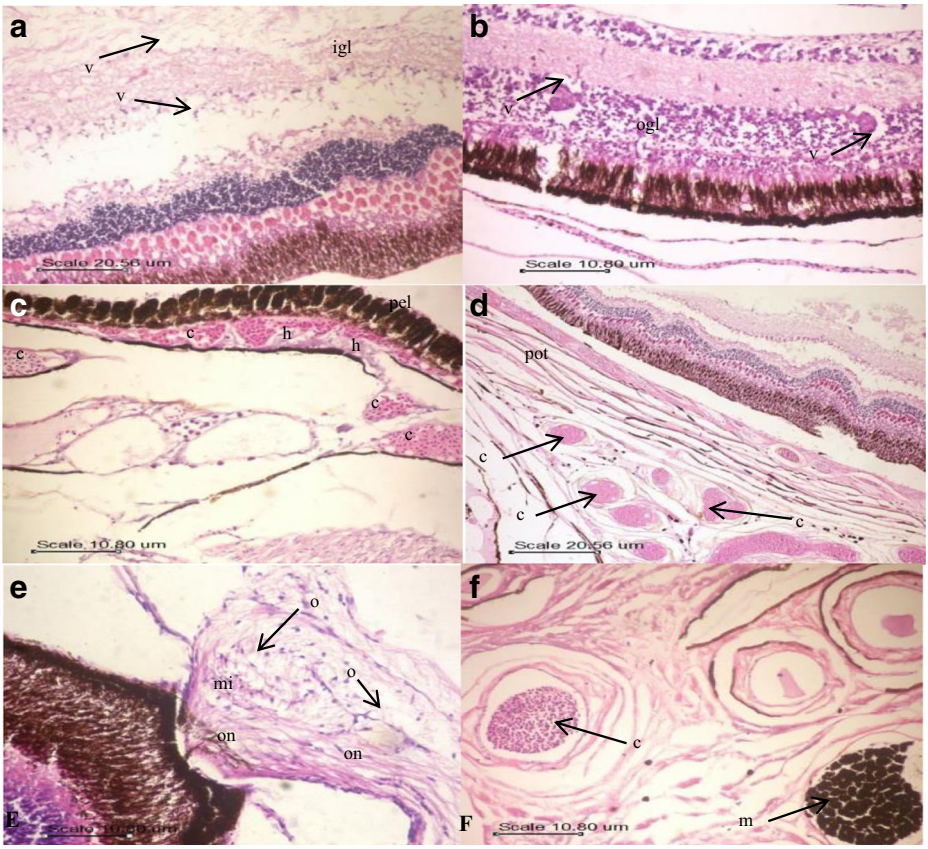

Fig. 3 Histopathological examination (H&E stain) of eye of diseased sobaity seabream *Sparidentex hasta* showing **a** severe vacuolation (*v*) of inner granular layer of the retina (*igl*) and oedema of nerve fibres. **b** Vacuolation (*v*) of outer granular layer (*ogl*) of the retina. **c** Sub-epithelial haemorrhage (*h*) and congestion (*c*) in the pigmented epithelium layer of the retina (*pel*). **d** Severe congestion (*c*) of blood vessels in the periorbital tissue (*pot*). **e** Vacuolation and oedema (*o*) of optic nerve (*on*) and mononuclear cells infiltration (*mi*). **f** Congestion (*c*) of the blood vessels in periorbital region with focal aggregation of melanophores (*m*)

congestion of the blood vessels in the brain, degenerative changes, mononuclear inflammatory cells infiltration and prominent eosinophilic intracytoplasmic inclusion bodies. The inclusion bodies could have resulted from viral replication, and many studies have shown that nodavirus replicates in the cytoplasm and forms paracrystalline array and inclusion bodies (Breuil et al. 1991; Delsert et al. 1997; Munday et al. 1992; Yoshikoshi and Inoue 1990).

Groupers are generally considered to be more susceptible to VNN, and many reports of NNV causing high mortalities in both young as well as adult groupers are available (OIE 2015; Kara et al. 2014). However, in the present case, brown-spotted groupers did not show any clinical signs or mortality even though subsequent tests revealed positive for NNV. Some earlier studies have already shown that adult fishes infected with nodavirus may not necessarily develop the disease (VNN). Age and temperature play a significant role in VNN disease development (Aranguren et al. 2002). It has been established that the temperature plays a vital role in the switching of asymptomatic carrier fishes and subsequent development of VNN

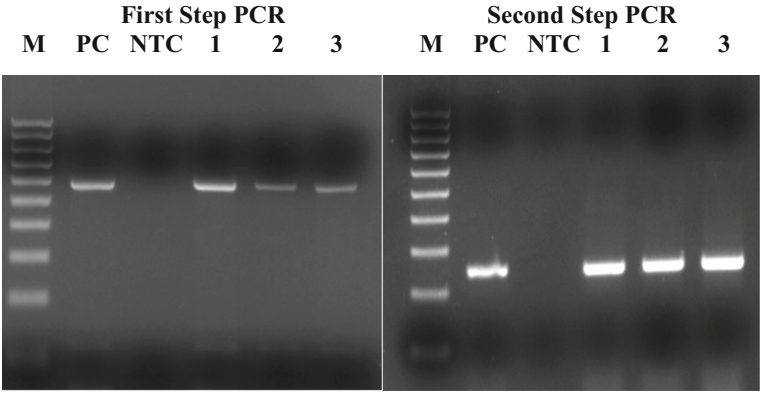

Fig. 4 Ethidium bromide stained agarose gel showing amplicon of first step (605 bp) and second step (254 bp) PCR. *Lane M*: marker (100 bp); *lane NTC*: no template control; *lane PC*: positive control; *lanes 1*, *2 and 3* are the amplicons of NNV detected in fries of gilthead seabream *Sparus aurata* and adults of sobaity seabream *Sparidentex hasta* and brown-spotted grouper *Epinephelus chlorostigma*, respectively

(Fukuda et al. 1996; Kara et al. 2014). Earlier reports also suggest younger fishes are more susceptible than the adults among various species. This may be because of lower and undeveloped immunity in young fishes (Johansen et al. 2002; Azad et al. 2005). In the present study, even though adults of brown-spotted grouper did not show any mortality, samples were detected positive in first step PCR showing these adults carry virus asymptomatically. Highly developed immune system of the bigger size fishes (all groupers were more than 4 kg in weight) or optimum environmental conditions (for fishes) may be the reason for non-development of the disease. However, the genetic variant of NNV may also be the reason. Present study showed that the infected samples showed a C_T value ranging from 17 to 22. Kuo et al. (2011) have concluded in there study that the real-time assay showing a C_T value of less than 24 to be positive for the NNV infection and C_T value ranging from 24 to 29 to be indication of suspected infection of NNV. Similarly, Hodneland et al. (2011) have also observed that the experimental VNN death of seabass had a C_T value of an average of 15.

Betanodavirus infection can spread both horizontally and vertically (Gomez et al. 2010; Johansen et al. 2004; Kai et al. 2010). In the present case, the source of infection is not clear and various route of virus entry is suspected. Imported gilthead seabream were not screened for the viruses and may be one of the possible route by which virus would have gained entry. Water used in the hatchery was not disinfected and was drawn directly from the sea which may be also a source of contamination. Piscine nodavirus can survive long in sea water (Frerichs et al. 2000; Mori et al. 1992), and any outbreak of VNN in the surrounding area can also be a source of NNV. Virus-contaminated trash fishes used as feed can also be a source. Trash fish as a source of *Betanodavirus* infection has been recorded in the Philippines and Japanese aquaculture systems (de la Peña et al. 2011; Gomez et al. 2010). Carrier wild fishes or other vectors which may have accidentally gained entry into the rearing unit along with the water may also be a reason for the outbreak.

Presently, there is no effective treatment available for nodavirus infection. Even though there are some successes in vaccination studies conducted (Kai and Chi 2008; Lin et al. 2007;

Pakingking et al. 2010), there are no commercial available vaccines for VNN. Hence, regular health monitoring and good management practises can help in avoiding the infection. Results of the present study are significant as it provides proof of the extent of spread of *Betanodavirus* both in species as well as geographical location within the Arabian Gulf.

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