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Nodaviruses in Wild Fish Population Collected Around Aquaculture Cage Sites from Coastal Areas of Tunisia

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Abstract

This report describes the viral epidemiology of wild fish adjacent to cage farms within the Tunisian coasts and is focused on viral nervous necrosis virus (VNNV). A total of 92 apparently healthy wild marine fish were collected near aquaculture facilities in five different coastal areas of Tunisia. The brains and eyes of fish were examined by quantitative real time reverse transcriptase-polymerase chain reaction (qRT-PCR) to detect the nodavirus coat protein gene of. A total of 57 out of 92 (61.9%) samples were positive for nodavirus by qRT-PCR. This finding indicates that carrier fish occur at a considerable level in populations of wild marine fish. Samples from 13 fish species were found to be positive to the virus genome: *Sarpa salpa, Trachurs trachurus, Boobs boops, Sardinella aurita, Diplodus vulgaris, Diplodus puntazzo Liza aurata, Diplodue sargus, Sparus aurata, Sardina pilchardus, Spicara maena, Spondyliosoma cantharus*, and *Diplodus annularis*. The partial sequences of the RNA2 coat protein gene of these strains were identical with RGNNV type previously identified within farmed sea bass and sea bream species in Tunisia, with a homology >97%. With respect to the proximity of the sampling sites to the coast and to rearing facilities, results analysis can suggest that these viruses may be indigenous to Tunisian coastal waters.

Keywords Wild fish; Piscine Nodavirus; Horizontal transmission; RT-qPCR

Introduction

The rise of novel forms of intensive aquaculture, the increased global movement of aquatic animals and their products, and various sources of anthropogenic stress to aquatic ecosystems have led to the emergence of many new fish diseases [1]. The use of feral brood stock is arguably the most significant biosecurity risk in aquaculture, as these fish can be covert carriers of viral pathogens [2]. Infectious disease emergence is also a concern in the wild fisheries and may occur due to the environmental pressures, the direct impact of human activities and the risk of pathogen spread from aquaculture [3]. In the present study, we carried out a preliminary epidemiological investigation concerning the zoo sanitary statute of wild fish population swimming in or around fish farming cages, to evaluate the potential impact of NNV transmission between wild and cultured fish. The virological survey was performed on several species of apparently healthy wild marine fish species as well as farmed sea bass. The aim of the project was to focus on the detection of healthy carriers of piscine Nodavirus due to the importance of this virus in Mediterranean and the North African aquaculture [4]. Indeed, viral nervous necrosis (VNN) has emerged to become a major problem in the culture of larval and juvenile marine fish worldwide [5]. Nodaviruses are a group of small viruses, non-enveloped, with a genome consisting of two molecules of singlestranded positive-sense RNA. The RNA strand is split into two sections: RNA1 and RNA2. RNA1 encodes an RNA-dependent RNA polymerase, whereas RNA2 encodes the coat protein gene [6]. At least five genogroups are recognized: red-spotted grouper nervous necrosis virus (RGNNV), striped jack nervous necrosis virus (SJNNV), barfin flounder nervous necrosis virus (BFNNV), tiger puffer nervous necrosis virus (TPNNV) and turbot nervous necrosis virus (TNNV) [7-9]. In the Mediterranean, Nodaviruses have been isolated from a number of wild and farmed fish species, including European sea bass *Dicentrarchus labrax (L.)*, for which VNN is a major constraint in hatcheries [10-14]. In the Mediterranean, Nodaviruses from the RGNNV, SJNNV groups as well as reassortants have been detected [15-17]. Phylogenetic analyses [18-20] have revealed that genetic lineages of the Nodaviruses show low host specificity and generally correspond to geographic location. This finding suggests that genotypes have generally emerged due to spill-over from reservoirs that include a broad range of wild marine fish, although some isolates revealed links to commercial movement [4]. Currently, there exists insufficient epidemiological evidence to support the listing of VNN as endemic to fish along the Tunisian coast line. To our knowledge, this is the first investigation performed in order to clarify the role of wild fish in the epidemiology of emerging viruses in North Africa aquaculture facilities. The study does not consider the disease, only the detection of the viral genome by reverse transcription-polymerase chain reaction (RT-PCR).

Material and Methods

Fish samples

The surveillance and sampling of wild fish populations collected near mariculture facilities situated at the north and east costs of Tunisia included 5 sites (A, B, C, D and E). For this purpose, 13 species of wild fish were investigated for the presence of the Nodavirus RNA2 segment during a routine survey conducted between January-July 2014. Samples description is summarized in Table 1.

Tissue sampling

Immediately after sampling, fish were stored at -20°C until used. For tissue analysis, fish were thawed and measured. Based on the size of the fish, those measuring less than 5 cm long, fish heads were entirely homogenized.

Species	Family	No of Individuals
Sarpa salpa	*Sparidae*	2
Trachurs trachurus	*Carangidae*	16
Boobs boops	*Sparidae*	17
Sardinella aurita	*Clupeidae*	6
Diplodus vulgaris	*Sparidae*	2
Diplodus puntazzo	*Sparidae*	1
Liza aurata	*Mugilidae*	4
Diplodue sargus	*Sparidae*	1
Sparus aurata	*Sparidae*	7
Sardina pilchardus	*Clupeidae*	27
Spicara maena	*Centracanthidae*	4
Spondyliosoma cantharus	*Sparidae*	4
Diplodus annularis	*Sparidae*	1
Total Number		92

Table 1: Number and characteristics of wild fish specimens examined in the study.

For specimens measuring more than 5 cm, fish were dissected and specific organs (brain, eyes) were harvested. Specimens were processed individually and homogenized in a sterile mortar with pestle and sterile sea sand then diluted to 1:10 (w: v) in Glasgow Minimum Essential Medium containing penicillin (200 International Units [I.U.]/ml), streptomycin (0.2 mg/ml) and kanamycin (0.2 mg/ml). The homogenates were then centrifuged at 4000 × g for 15 minutes at 4°C and supernatants were stored at -80°C.

Genome extraction and virus detection by amplification

RNeasy Mini Kits (Qiagen) were used to purify total RNA from organ homogenates according to the manufacturer's instructions. RNA elution was performed in a final volume of 30 µL of RNase-free water before storage at -80°C. RNA quantities and purity (260/280 ratio) were estimated by spectrophotometry (Nano drop ND-2000, Thermo scientific).

Nodavirus screening was performed by qRT-PCR using the Applied Bio system 7500 machine and reaction conditions were realized according the validated protocol of Panzarin et al. [21] with a slight modification of hybridization and extension duration which was adaptable to the 7500 machine. Oligonucleotides were able to target and amplify a conserved 69-bp-long region of the viral genome localized in the RNA2 strand, encoding the coat protein (CP). The sequences of the primers and probe are as follows: RNA2 FOR 5'-CAACTGACARCGAHCACAC-3', RNA2 REV 5'-CCCACCAYTTGGCVAC-3', RNA2 TaqMan probe 5'-6FAM-TYCARGCRACTCGTGGTGCVG-BHQ1-3'The final 25 µL reaction volume contained 0.6 µM of each primer and approximately 100 mg of total extracted RNA as template. Positive and negative controls were added in each step of the amplification. The thermal conditions that were applied consisted of a 10-min incubation at 95°C followed by 45 cycles of 10 s denaturation at 95°C, 35 s annealing at 58°C and 30 s elongation at 72°C, followed by an additional 30 s cooling step at 40°C.

Analysis of sequence data

Amplified products were purified by using a High Pure PCR products purification kit and were sequenced in both orientations by using an ABI 373A automated sequencer (Perkin Elmer) and an ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer), following the manufacturer's instructions. BLASTN analysis was conducted with representative virus sequences exhibiting significant sequence. Partial RNA2 nucleotide sequences of 32 Nodavirus isolates available in GenBank were included in the analyses. Pair wise comparison was performed using the blast2seq program. Nucleotide partial sequences of the MCP gene were aligned via the MEGA6 [22] software using the Muscle method, and final adjustments were performed manually. The phylogenetic tree was constructed using the MEGA6 with UPGMA method and the final phylogenetic tree was drawn with the Coral DRAWX6 program. Because the sequences from GenBank were heterogeneous in length, the longer sequences were cut and adapted to the shortest. The reliability of the tree was inferred using the bootstrap method with 1000 replicates [23]. The partial nucleotide sequences of the Nodavirus strains screened in the present study were not deposited in GenBank because they are less than 200 pb.

Results

A total of 92 wild fish samples composed of 5 families and 13 species were collected and processed (Table 1). No clinical signs were observed among the fish tested. Approximately 62% (57 fish) of the samples were found positive for the viral genome by real time RT-PCR. When comparing all the sampling points the lowest apparent prevalence was detected in February 2014 (28%) and the highest was of 100% detected in July 2014. All species analysed were positive, although apparent prevalence rates differed due to the difference in the collected number of samples monthly (Table 2). Positive results were obtained in the brains and eyes of the following fish: *Sarpa salpa, Trachurs trachurus, Boobs boops, Sardinella aurita, Diplodus vulgaris, Diplodus puntazzo, Liza aurata, Diplodue sargus, Sparus aurata, Sardina pilchardus, Spicara maena, Spondyliosoma cantharus*, and *Diplodus annularis*. Apparent prevalence of Nodavirus within the different fish families showed that the *Sparidae* and the *Carangidae* families present a great susceptibility to the virus (with a detection rate of approximately 92% and 60%, respectively).

Comparison of a partial sequence from the coat protein gene of the wild isolates showed a similarity ranging from 94% to 97% to the Tunisian nodaviruses retrieved from farmed sea bass and sea bream [4,24]. In addition, the similarity with the rest of the genotypes was ranging from 88% to 97% (data not shown). All of the Tunisian isolates from farmed fish are classified as RGNNV genotype [4] however isolates from wild fish sequenced in this study could not be grouped with the RGNNV (Figure 1) unless further amplifications targeting variable RNA2 region will be conducted.

Site	Month	Nature*	Family	Species	Fish N°	qRT-PCR
A	Jan	W		*Trachurs trachurus*	3	+
A	Feb	W		*Trachurs trachurus*	3	-
A	Apr	W	*Carangidae*	*Trachurs trachurus*	3	-
A	Apr	W		*Trachurs trachurus*	3	+
A	May	W		*Trachurs trachurus*	3	+
Total des Carangidae					15	Sep-15
A	Jan	W		*Boobs boops*	3	+
A	Feb	W		*Boobs boops*	4	+
B	Apr	W		*Boobs boops*	4	+
A	Apr	W		*Boobs boops*	1	+
A	May	W		*Boobs boops*	1	+
C	Jul	W		*Boobs boops*	9	+
A	Jan	W		*Sarpa salpa*	1	-
A	Feb	W		*Sarpa salpa*	1	-
A	Jan	W	*Sparidae*	*Diplodus vulgaris*	1	+
A	May	W		*Diplodus vulgaris*	1	+
D	May	W		*Diplodus vulgaris*	2	+
D	May	W		*Diplodus sargus*	1	+
D	May	W		*Diplodus puntazzo*	1	-
C	Jul	W		*Diplodus annularis*	1	+
C	Jul	W		*Spondyliosoma cantharus*	4	+
Total of Sparidae					35	32/35
E	Feb	W		*Sardina pilchardus*	5	-
E	Feb	W		*Sardina pilchardus*	4	-
E	Feb	W		*Sardina pilchardus*	5	+
E	Feb	W		*Sardina pilchardus*	5	-
E	Feb	W		*Sardina pilchardus*	4	-
A	May	W		*Sardina pilchardus*	4	+
E	Feb	W	*Clupeidae*	*Sardinella aurita*	1	+
A	Jan	W		*Sardinella aurita*	1	-
Total of Clupeidae					25	Oct-25
D	May	W		*Liza aurata*	2	+
D	May	W	*Mugilidae*	*Liza aurata*	2	-
Total of Mugilidae					4	02-Apr
C	Jul	W	*Centracanthidae*	*Spicara maena*	4	+

Total of Centracanthidae	4	04-Apr
Total of wild fish =	92	57/92

Table 2: Sampling details including dates, sites, nature of the caught fish (W=wild), fish family and species; in addition to the qRT-PCR results targeting Nodavirus RNA2 segment.

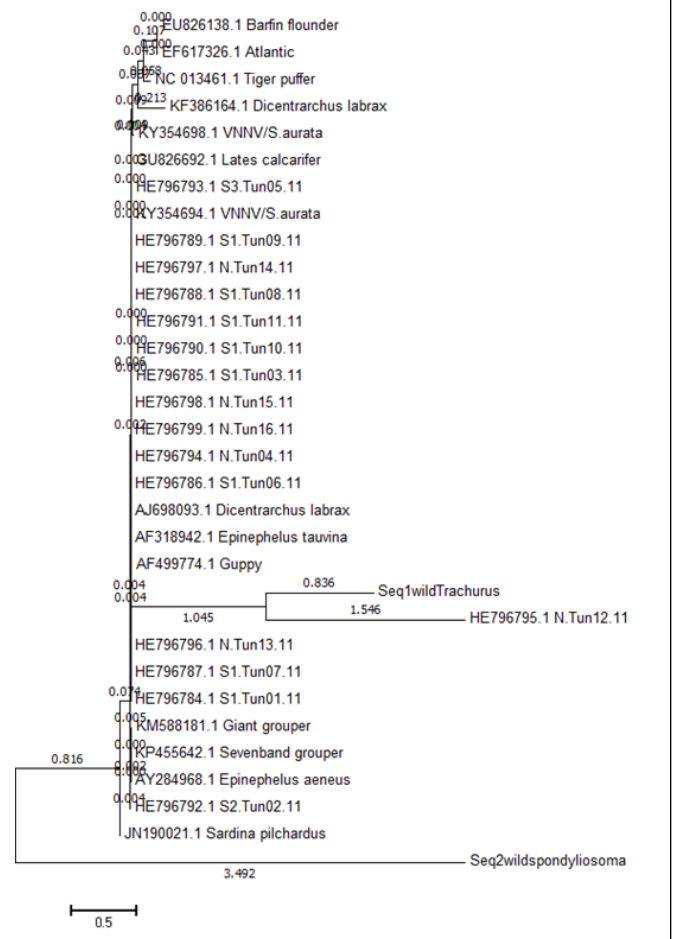

Figure 1: Phylogenetic relationships between Nodavirus isolates based on a partial nucleotide sequence of the capsid protein (CP) gene.

The evolutionary history was inferred using the UPGMA method [25].The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [23]. The evolutionary distances were computed using the Kimura 2-parameter method [26] and are in the units of the number of base substitutions per site [27-30]. GenBank accession numbers and references for nucleotide sequences are as follow: EU826138.1[31], EF617326[32], NC_013461[33], KF386164[34], KY354698.1[35], GU826692.1[36], HE796793.1[37], KY354694.1[35], HE796789.1[37], HE796797.1[37], HE796788.1[37], HE796791.1[37], HE796790.1[37], HE796785.1[37], HE796798.1[37], HE796799.1[37], HE796794.1[37], HE796786.1[37], AJ698093.1[38], AF318942.1[39], AF499774.1[40], HE796795.1[37], HE796796.1[37], HE796787.1[37], HE796784[37], KM588181.1[41], KP455642.1[42], AY284968.1[43], HE796792.1[37], JN190021.1[44].

Discussion

To date, nodavirus has been isolated from approximately 50 fish species worldwide and several studies have established that this virus is globally distributed in the environment [15,19,20]. In addition, the number of possible host species is increasing with increasing monitoring efforts. The most susceptible farmed fish species in the Mediterranean is sea bass, although nodavirus is also reported to cause mortality in farmed sole [25]. Among wild fish, important outbreaks have been observed in recent years in south Italy and Algeria involving at least 2 species of grouper. Nodavirus isolates from these outbreaks belong to genotype RGNNV [26,27].

For the fish populations analysed in this study, phylogenetic analysis could not be achieved due to the nature of the target amplified region. However, RGNNV genotype is probably the only genotype circulating within the Tunisian coasts actually. This is concordance with results obtained by Vendramin et al. who detected only RGNNV in wild fish collected in southern Italy [26]; however, López-Jimena et al., detected RGNNV and SJNNV genotypes from wild asymptomatic meagre specimens (*Argyrosomus regius*) which were caught in the Atlantic coast of the Iberian Peninsula [29].

The detection of nodavirus in Tunisia occurred several years ago in farmed sea bass and sea bream [4-20]. In this study, the causative agent was detected from feral fish species for the first time. To our best knowledge, this is the first report of VERV infection in these fish species in Tunisia from coastal waters. In fact, the newly obtained Tunisian sequences from RNA2 were resized and compared with a large range of sequences extracted from GenBank, high levels of similarity (>94%) were found with RGNNV isolates from various fish from Tunisia and different geographical origins [15,18,20]. Interestingly, the sequences from this study were highly similar to isolates from reared sea bass from Tunisia, found in over the past 10 years. This result strongly suggests that transmission of the virus between these groups probably occurs and that this virus is likely endemic along the Tunisian coast. Although the correlation between infection status of nearby fish farms and evidence of infection in free living species has been established; the direction of viral transmission (i.e. farm to wild or wild to farmed fish) is often difficult to ascertain.

The exchange of pathogens between farmed and wild fish has been repeatedly observed and investigation results indicated that marine strains are not without significance to the farmed fish industry. The healthy carrier species, although asymptomatic but also migratory, could induce the spread of virus to other fish cage in other locations. Unfortunately, in the present study, it was not possible to characterize properly the new isolates due to the short sequenced region (<200 bp) or to trace their origin owing to its high genetic similarity to isolates from very diverse origins, from Mediterranean countries or from Asia.

However, the importance of the screening of new viral reservoirs done in this study concerns the fact that the environmental conditions play a key role in switching fish from a carrier state to an acute phase. Indeed, it is known that temperature is a major factor for stimulating asymptomatic wild carriers to become potential sources of the virus for other species, including farmed fish such as sea bass and sea bream. Moreover, exotic viruses trans-located into new geographic areas have spread to cause major disease outbreaks in populations of both native and cultured species and the trans-boundary movement of aquatic animals continues to be one of the greatest threats to the productivity and profitability of aquaculture worldwide [45-47]. Thus, further intensive molecular epidemiological, pathological and virological analyses will be required.

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Conflict of Interest

The authors declare that they have no conflicts of interests.

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