ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA
DIPARTIMENTO DI SCIENZE MEDICHE VETERINARIE

DOTTORATO DI RICERCA in Scienze Veterinarie
XXXV CICLO - A.A. 2019/2020
CURRICULUM: Sanità animale
Anno di attività: [3°]
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Development of a fluorescent *in situ* hybridization technique to detect the Nervous Necrosis Virus in aquatic animal tissues

Introduction

The Nervous Necrosis Virus (NNV) is considered one of the most threatening pathogens for Mediterranean aquaculture and it is responsible of a neuropathological condition described in at least 62 marine species (Bandin and Souto, 2020). Furthermore, the presence of NNV has been reported in several invertebrate species, including bivalve mollusks. These animals are able to accumulate and release viable particles of NNV in the environment (Volpe et al., 2017) but at present their epidemiological role is not fully understood as the replication of NNV within their tissues has not been reported yet.
The fluorescent *in situ* hybridization technique (FISH) consists in a sensitive method able to target specific nucleic acid sequences (DNA or RNA) at cellular level and potentially is able to correlate the presence of viruses to tissues alterations and recognize infections in sub-clinical cases (Pfankuche et al., 2018). The aim of this study was to design specific RNA probes to detect the presence of NNV genome and the complementary RNA (cRNA), produced during viral replication, in aquatic animal tissues including bivalve mollusks. The method was developed in order to localize NNV within the tissues and clarify the kind of relationship between the virus and bivalve mollusks.

M&M

FISH was performed using two self-designed but commercially produced probe sets: a sense probe able to detect NNV genome (TYPE 1 probe) and an anti-sense probe able to visualize the cRNA produced during viral replication (TYPE 6 probe). Probes were designed on a conserved region of NNV genome that encodes for RNA polymerase, choosing two sequences of approximately 1000 nucleotides each with a balanced GC-content (**Fig. 1**).
The optimal conditions to perform FISH were established on striped snakehead cell pellets (SSN-1), experimentally infected with NNV. Subsequently, the optimized method was applied to clam samples.

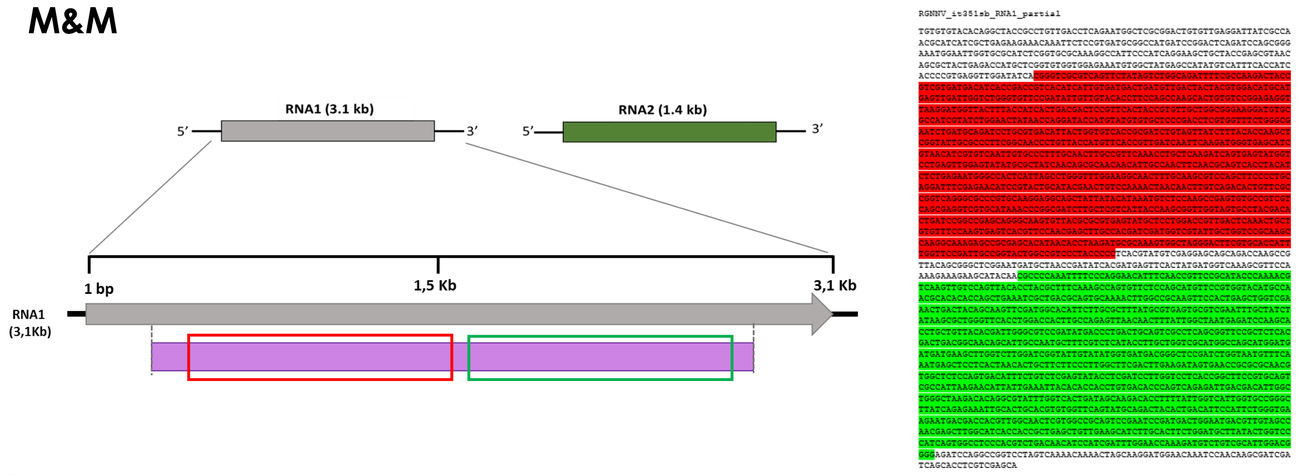

Fig. 1 *Conserved NNV region used for probe design. Red box represents the area chosen for the TYPE 1 probe, that detects NNV genome; Green box represents the area chosen for the TYPE 6 probe that detects the complementary RNA produced during viral replication*

Results

NNV-infected cell pellets showed strong positive signals visible as red and green signals for TYPE 1 and TYPE 6 probes respectively (**Fig. 2**).
Clam samples showed clear signals only in the ovarian tissue. Particularly, red and green signals were diffusely distributed in the citoplasm of cells. Furthermore, areas with overlapping of probe signals were present. Areas with the overlapping signals appeared as yellow (**Fig. 3**). The negative controls, including non-infected SSN-1 cell pellets and samples without probe application, did not show specific signals.

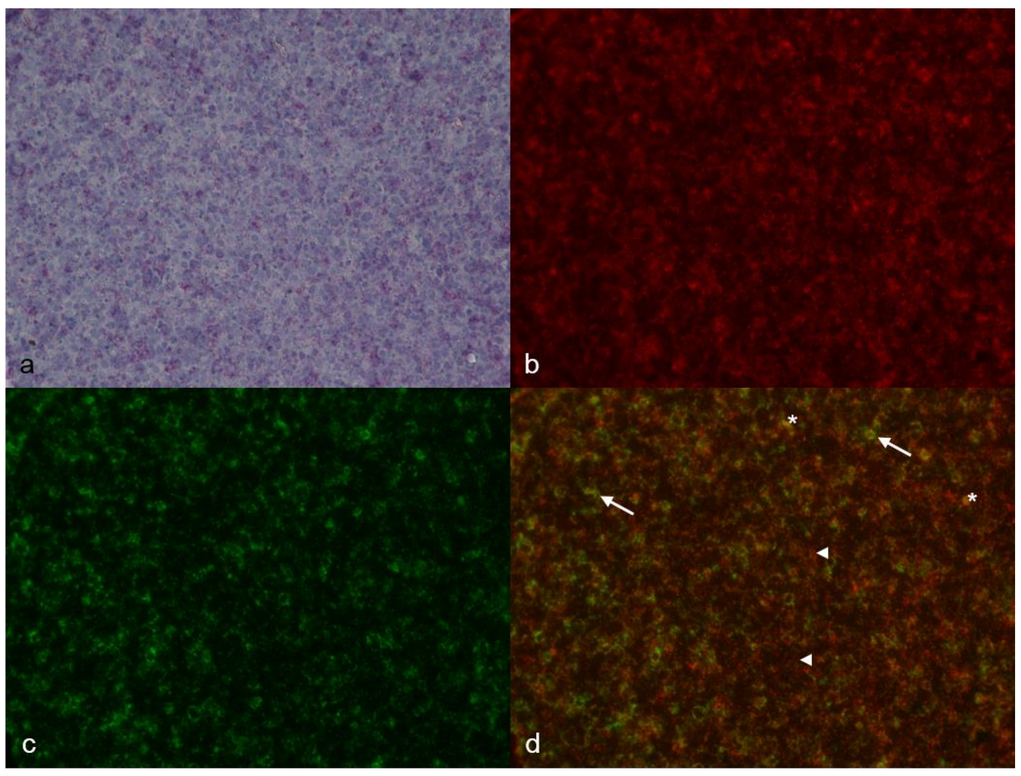

Fig. 2 *2-plex fluorescent* in situ *hybridization results for NNV infected SSN-1 cells. (a) SSN-1 cell pellet, Bright-field 40x. (b) Hybridization with TYPE 1 probe targeting NNV genome (Red), 40x. (c) Hybridization with TYPE 6 probe targeting the cRNA produced during viral replication (Green), 40x. (d) Overlay of (b) and (c) shows areas of cells exclusively labelled with each of the two probes (Red: arrowheads; Green: arrows), and areas with overlapping probe signals, resulting in yellow (asterisks), representing areas with the co-expression of both targets of interest, 40x.*

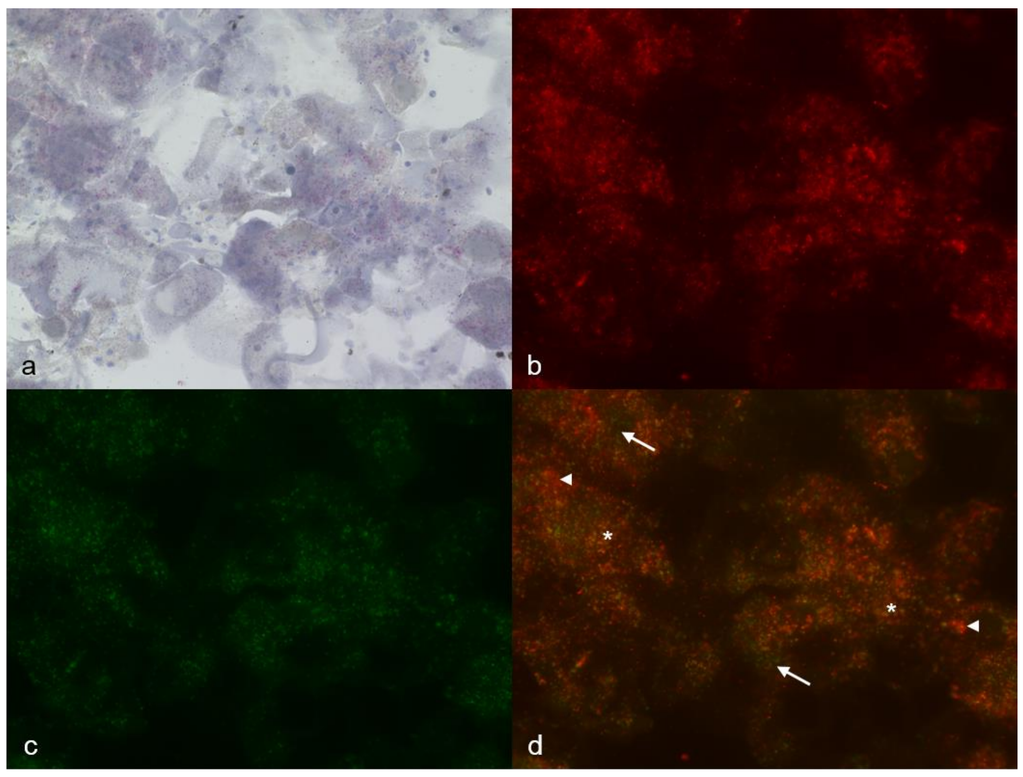

Fig.3 *2-plex fluorescent* in situ *hybridization results for clams. (a) Clam ovarian tissue, Bright-field, 40x. (b) Hybridization with TYPE 1 probe targeting NNV genome (Red), 40x. (c) Hybridization with TYPE 6 probe targeting cRNA produced during viral replication (Green), 40x. (d) Overlay of (b) and (c) shows areas of cells exclusively labelled with each of the two probes (Red: arrowheads; Green: arrows), and areas with overlapping probe signals, resulting in yellow (asterisks), representing areas with the co-expression of both targets of interest 40x.*

Conclusions and Future Proposal

The developed method was able to detect the presence of NNV genome in the cytoplasm of cells and the complementary RNA produced during viral replication. Particularly, the expression of both targets of interest was clearly seen in the NNV-infected SSN-1 cell pellets and in clams' ovarian tissue. Areas with overlapping of probe signals are presumed to represent areas with viral replication.
Concluding, the developed 2-plex FISH showed the specificity of the designed probes for the detection of NNV targets in aquatic animal tissues of different origin (SSN-1 cells and clam samples) and allowed to clarify the localization of the virus within cells and sites of replication. Furthermore, the 2-plex FISH represents an efficient method that can be used in future application to improve the knowledge on the epidemiology and the pathogenesis of this disease.

References

- Bandin I., and Souto S. (2020) Betanodavirus and VER Disease: A 30-year Research Review. Pathogens, 9, 106.
- Volpe E., Pagnini N., Serratore P. and Ciulli S. (2017) Fate of redspotted grouper nervous necrosis virus (RGNNV) in experimentally challenged Manila clam *Ruditapes philippinarum. Diseases of Aquatic Organisms, 125: 53-61.*
- *Pfankuche V. M., Hahn K., Bodewes R., Hansmann F., Habierski A., Haverkamp A. H., Pfaender S., Walter S., Baechlein C., Postel A., Steinmann E., Becher P., Ostrehaus A.,* Baumgärtner W. and Puff C. (2018) Comparison of Different in situ Hybridization Techniques for the Detection of Various RNA and DNA viruses. Viruses, 10, 384.