EVALUATION OF *IN VITRO* VIRUCIDAL ACTIVITY OF VIRKON®S AGAINST *BETANODAVIRUS*

Sara Ciulli, Enrico Volpe, Marco Grodzki

Department of Public Veterinary Health and Animal Pathology, Alma Mater Studiorum – University of Bologna, Faculty of Veterinary Medicine, Degree Course in Aquaculture and Hygiene of Fish Productions

INTRODUCTION

Betanodavirus causes an infectious disease in many marine fish species, responsible of nervous signs and mortality, with consequent high economic losses. From the economical point of view, the sector mostly hit in Italy by Viral Encephalo-Retinopathy (VER) is Sea bass farming (*Dicentrarchus labrax*) that accounts for the majority of fish productions. The stages that are mostly involved are larvae and fry, though in Sea bass it's possible to observe outbreaks also in adult, commercial size fish. There is no therapy and no vaccines at the moment that permit an adequate control of this disease, even though recent *in vitro* trials performed in the laboratories of the Department of Veterinary Public Health and Animal Pathology at the University of Bologna were able to prove the antiviral activity of some molecules towards *Betanodavirus*.

Therefore, the disease control is at the moment based on maintaining correct hygienic-sanitary procedures and correct livestock management, with a particular attention to biomass density.

The use of direct prophylaxis for the control of infectious diseases requires a deeper knowledge of characteristics of pathogens resistance and the availability of products and disinfection protocols for the control of pathogens.

In order to enlarge the knowledge on efficacy of disinfectants towards *Betanodavirus*, *in vitro* trials were performed in this survey, to evaluate virucidal activity of a commercial product that is already used in veterinary medicine.

To confer international validity to tests of evaluation of the disinfectant's virucidal activity, the assay was done according to BS EN 14675:2006 protocol, acknowledged as European standard protocol.

Materials and Methods

Virus

The virus used in this trial was isolated from European Sea bass fry (*Dicentrarchus labrax*) during a natural outbreak of VER in the north Adriatic Sea (Ciulli *et al.*, 2006). The virus was isolated using SSN1 cell line (Frerichs *et al.*, 1996) and called IT/351/Sb. After genetic characterization, the virus resulted belonging to RGNNV species, Genus *Betanodavirus*, Family *Nodaviridae.* This viral species is commonly isolated during Viral Encephalo-Retinopathy outbreaks in European Sea bass and also in other fish species in the entire Mediterranean Sea. The RGNNV virus was also discovered in many wild fish species in the same area (Ciulli *et al.*, 2007).

Cell line

The cell line we used is the SSN1 cell line. The cells were cultured in L-15 nutrient medium (Leibovitz medium - Invitrogen) to which an antibiotic solution and L-glutamine were added, both at 1% v/v concentration, furthermore bovine fetal serum (BFS) as a protein source was added, at 7,5% v/v or 2% v/v concentration, as needed.

Virkon®S

The Virkon®S disinfectant (DuPont Wilmington, DE) was tested at 1% p/v and 2% p/v concentrations following the manufacturer's indications. As recommended by BS EN 14675:2006 protocol, the diluent used was hard water (HW; 1.248mM $MgCl_2$, 3.328mM $CaCl_2$, 2.496mM $NaHCO_3$).

The dilutions were prepared at the beginning of each trial dissolving 0,125 g (1,25%) and 0,250 g (2,5%) of the product in 10 ml of HW. The concentrations were increased in order to obtain the real exposition of the virus to the dose of disinfectant recommended by the manufacturer.

Viral titration

The produced virus was titrated on 96-well plates according to the dilution method for the calculation of $TCID_{50}$/ml (50% Tissue Culture Infective Dose) described afterwards. The calculation of the dose infecting 50% of the cultured tissue ($TCID_{50}$/ml) was performed according to the Reed & Muench method (1938).

The protocol of evaluation of virucidal activity of disinfectants

All disinfectant's virucidal activity evaluation tests were carried out following the BS EN 14675:2006 protocol, set up by BSi-British Standards, and approved by CEN (Comité Européen de

Normalisation), becoming thus a standard of the European Community. Furthermore, this protocol is followed both by United Kingdom's Department for Environment, Food and Rural affairs (Defra), for drafting the list of disinfectants approved for veterinary use, and by the Norwegian Ministry of Agriculture.

The protocol is based on mammal viruses, thus some modifications were done, to adapt it to fish viruses according to guidelines published by Verner-Jeffreys *et al.* in 2009. Verner-Jeffreys *et al.* (2009) modified the protocol in order to adapt it and assess the virucidal activity of some disinfectants against the Infectious Salmon Anaemia Virus (ISV); in particular, the modifications involved lowering the exposure time to the disinfectant, the use of marine water as diluent and adding an inhibitory substance (bovine albumin fraction V, 3 g/100 ml) as organic matter substitute. In our case, times and temperature of exposition were modified in order to adapt the protocol to the evaluation of Virkon®S's virucidal activity against *Betanodavirus.*

This protocol, applied to ISA virus, was used as well to test virucidal activity of disinfectant substances against the virus of pancreatic disease (PD) or the "sleeping disease" of Salmon (Graham *et al.*, 2007). The reason for the few tests against viruses pathogenic to fish species, carried out following the BS EN 14675:2006 protocol, is due to recent publication of the protocol itself.

The virucidal activity is calculated subtracting the viral titre observed after the treatment from the viral titre obtained without any treatment (virus control), in this way we achieve a logarithmic base result called relative reduction (R). A disinfectant, to be considered efficacious according to BS EN 14675:2006 protocol, must achieve a relative reduction of at least 4 logarithms ($R \geq 4$).

The trials were conducted in 100 µl volumes, with a 1:1:8 ratio of, respectively, virus, inhibitor (bovine albumin, fraction V, 3 g/100 ml) and disinfectant, as recommended by BS EN 14675:2006 protocol; each disinfection trial included a virus control where, instead of the disinfectant, a diluent was used. Elapsed the tested exposure contact time, the disinfectant's action was stopped according to a dilution procedure. Furtherly, residual viral titre of the treated solution and that of the control was measured.

Regarding Virkon®S, 1% p/v and 2% p/v dilutions were tested, with 1, 5, 10 and 20 minutes exposure time; the latter was tested only at a temperature of 10°C, whereas all the other exposure times were tested both at 20°C and at 10°C.

The readings of culture plates were done using an inverted microscope, 7 days after the disinfection trial. The presence of cytopathic effect was considered as positivity in the well, independently from the extension of the effect itself, as described in 50% tissue culture infective dose ($TCID_{50}$/ml) calculation method, according to Reed & Muench (1938).

Results

Viral titration of the produced viral stock, conserved in aliquots at -20°C, reported a value of 10^7 $TCID_{50}$/ml.

The 96-well plates used in residual infectivity tests of the virus treated with the disinfectant were read at inverted microscope, evaluating the presence/absence of the cytopathic effect. Different dilutions of the disinfectant mixture and of the virus used in the tests led to different grades of cytopathic effect.

The results of residual infective activity titration trials after the disinfectant treatment were expressed as $TCID_{50}$/ml. According to Reed & Muench method (1938), calculation of positive wells, showing cytopathic effect was made, independently from the entity of the effect itself, and calculation of $TCID_{50}$/ml, using Excel calculation sheet.

The virucidal activity resulting from the trials was expressed as relative reduction (R) calculated subtracting viral titre, obtained after the treatment, from viral titre achieved without treatment (virus control).

A disinfectant, in order to be considered efficacious, according to BS EN 14675:2006, must achieve a relative reduction of at least 4 logarithms ($R \geq 4$).

Tests of residual infectious activity evaluation after Virkon®S treatment never showed the presence of viral activity in the treated solution, but rather a toxic effect at first dilution. The presence of toxic effect that prevented the evaluation of presence or absence of cytopathic effect in this dilution led us to calculate $TCID_{50}$/ml values starting from the second dilution; similarly, also in virus controls, first dilution was not considered.

In the table, $TCID_{50}$/ml values obtained after a disinfecting treatment with Virkon®S are listed, flanked by $TCID_{50}$/ml value achieved from virus control; the difference between those two values is thus expressed as relative reduction.

Conclusions

In conclusion, this work made possible the successful acquisition of BS EN 14675:2006 protocol for the analysis of virucidal activity of disinfectants towards aquatic pathogens.

The application of this protocol permitted to evaluate the disinfectant activity of a commercial product which showed a high virucidal effect towards *Betanodavirus,* suggesting its possible, safe application also in marine aquaculture.

Time (min)	Temperature (°C)	Disinfectant concentration	Diluent	Replicas	$TCID_{50}$/ml after disinfection[a]	$TCID_{50}$/ml Virus control	Relative reduction
20	10	2%	HW	Test 1	0*	5,70*	5,70
				Test 2	0	5,20	5,20
				Test 3	0*	4,92*	4,92
10	20	1%	HW	Test 1	0*	6,07*	6,07
				Test 2	0*	5,53*	5,53
				Test 3	0*	5,87*	5,87
10	10	1%	HW	Test 1	0	6,53	6,53
				Test 2	0*	5,80*	5,80
				Test 3	0*	5,06*	5,06
5	20	1%	HW	Test 1	0	6,07	6,07
				Test 2	0	6,32	6,32
				Test 3	0	6,07	6,07
5	10	1%	HW	Test 1	0	7,02	7,02
				Test 2	0	6,70	6,70
				Test 3	0	7,02	7,02
1	20	1%	HW	Test 1	0	6,20	6,20
				Test 2	0	6,32	6,32
				Test 3	0	6,07	6,07
1	10	1%	HW	Test 1	0	6,20	6,20
				Test 2	0	6,32	6,32
				Test 3	0	5,87	5,87

Table: Results of evaluation of Virkon®S's virucidal activity.

[a] in presence of toxic effect at first dilution, $TCID_{50}$/ml values were read starting from second dilution (*)

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Summary

Betanodavirus is responsible of an infectious disease in many marine fish species, accounting for nervous signs and mortality, with consequent high economic losses. There is no therapy and no vaccines at the moment that permit an adequate control of this disease, even though recent *in vitro* trials tested successfully some molecules with antiviral activity.

Therefore, the disease control is at the moment based on maintaining correct hygienic-sanitary procedures and correct livestock management, with a particular attention to biomass density.

The use of direct prophylaxis for the control of infectious diseases requires a deeper knowledge of characteristics of pathogens resistance and the availability of products and disinfection protocols for the control of diseases.

In order to enlarge the knowledge on efficacy of disinfectants towards *Betanodavirus*, *in vitro* trials were performed in this survey, to evaluate their specific virucidal activity.

The analyzed product is already on sale in veterinary field and their biocidal and virucidal activity towards other pathogens is reported.

In particular, the activity of Virkon®S was inquired, a peroxides and organic acids-based disinfectant, the activity of which was tested towards a large number of bacteria and viruses of veterinary interest, with some references also to fish pathogens.

To confer international validity to performed tests and to obtain results that are comparable to those of the activity of other disinfectants and towards other microorganisms, BS EN 14675:2006 protocol was applied, set up by BSi-British Standards and approved by CEN (Comité Européen de Normalisation), acknowledged thus as European Community standard protocol.

Regarding Virkon®S, a powerful virucidal activity against *Betanodavirus* was observed.