ISTITUTO ZOOPROFILATTICO SPERIMENTALE DELL'UMBRIA E DELLE MARCHE "TOGO ROSATI"

3rd International Symposium on the Advances in Marine Mussel Research

Chioggia 26-28 Agosto 2019

HALOBACTERIOVORAX ISOLATED FROM MARINE WATER OF THE ADRIATIC SEA TO CHALLENGE *V.PARAHAEMOLYTICUS* IN *MYTILUS GALLOPROVINCIALIS*

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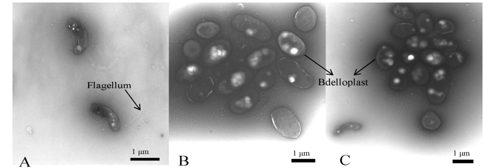
Background

V.parahaemolyticus strains producing thermostable direct haemolysin (TDH) and/or TDH-related haemolysin (TRH) are recognized as a cause of diarrhoeal diseases worldwide, with bivalves, eaten raw or undercooked being the most common sources of infection. The problem of mussel contamination by toxigenic *V. parahaemolyticus* kept growing, in the last decades, in Italy (Ottaviani et. al. 2010a, 2013). Moreover, illness due to *V.parahaemolyticus* with mussels or seawater of Adriatic as the source of infection, have been reported (Ottaviani et al., 2008, 2010b, 2012). Depuration is a very effective process for the elimination of faecal bacteria but it is less effective against naturally occurring *Vibrio* spp. (Andrews 2004). If on one hand conventional approach to the depuration of bivalves lacks in reducing *V. parahaemolyticus*, on the other innovative post-harvest treatments are expensive, kill bivalves and do not satisfy those consumers who prefer live bivalves (Andrews 2004). To increase the efficacy of conventional depuration towards *V.parahaemolyticus*, it should be possible to integrate it with forms of biological control. *Bdellovibrio* and like organisms (BALOs) are Gram-negative, aerobic bacteria which prey upon other Gram-negative bacteria; *Halobacteriovorax* genus includes the marine members of this group. In our previous study we isolated from seawater of the Adriatic Sea of Italy a *Halobacteriovorax* strain, named HBXCO1, that showed high predatory efficiency towards a wide range of pathogenic vibrios, including *V. parahaemolyticus* (Ottaviani et al., 2018).



Transmission electron micrograph of HBXCO1 (A) in the free attack phase of primary prey, showing solitary and flagellated cells (×13,000), (B) while replicating inside the bdelloplasto (x 10,000), (C) during the lysis of prey and release of progeny (x 8,000)

Aims:

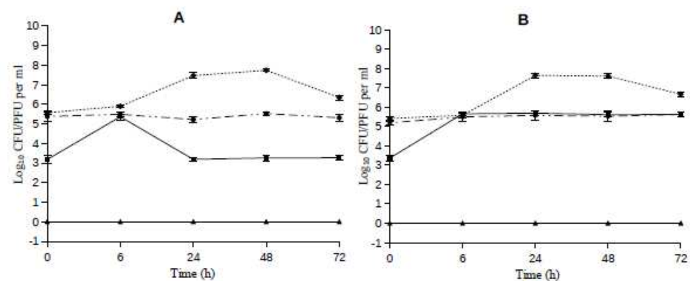
- To test predatory efficiency "in vitro" of HBXCO1 towards 17 Vibrio and 7 non-Vibrio strains.
- To evaluate the application of HBXCO1 in *Vibrio parahaemolyticus* infected mussel decontamination, at the laboratory scale

Prey strains Toxin genes *	Source Year of isolation	Origin	Susceptibility to HBXOC1
V.parahaemolyticus *tdh+**; trh -***; toxRS +* (pandemic strain)	feces 2007	Adriatic, Italy	+
V.parahaemolyticus *tdh-;trh+; toxRS-*	feces 2009	Adriatic, Italy	+
V.parahaemolyticus *tdh-/trh+;toxRS-*	marine water 2011	Adriatic, Italy	+
V.parahaemolyticus *tdh+/trh-;toxRS-*	mussels 2014	Mediterranean, Italy	+
V.parahaemolyticus *tdh-;trh-; toxRS-*	mussels 2015	Adriatic, Italy	+
V.parahaemolyticus *tdh-;trh+; toxRS*	mussels 2016	Adriatic, Italy	+
V.cholerae non O1/O139 *hly*AClass-; *hly*ET+;*ctx*A-;*tcp*AClass+; *tcp*AET-; *stn/sto-*	subcutaneous tissue 2009	seawater of Adriatic, Italy as the likely source of infection	+
V.cholerae non O1/O139 *hly*AClass-; *hly*ET+;*ctx*A-;*tcp*AClass-; *tcp*AET-; *stn/sto+*	marine water 2011	Adriatic, Italy	+
V.cholerae non O1/O139 *hly*AClass-; *hly*ET+;*ctx*A-;*tcp*AClass-; *tcp*AET-; *stn/sto-*	subcutaneous tissue 2012	seawater of Adriatic, Croazia as the likely source of infection	+
V.cholerae non O1/O139 *hly*AClass-; *hly*ET+;*ctx*A-;*tcp*AClass -; *tcp*AET-; *stn/sto-*	feces 2012	bivalves as the likely source of infection	+
V.cholerae non O1/O139 *hly*AClass-; *hly*ET+;*ctx*A-;*tcp*AClass-; *tcp*AET-; *stn/sto-*	mussels 2014	Adriatic, Italy	+
V.vulnificus nd ****	clams 2013	Adriatic, Italy	+
V.alginolyticus nd	mussels 2012	Adriatic, Italy	-
Salmonella napoli nd	mussels 2015	Adriatic, Italy	-
E.coli nd	mussels 2016	Adriatic, Italy	-
A.hydrophila nd	mussels 2004	Adriatic, Italy	-
V.parahaemolyticus NCTC 10885			+
V.cholerae non O1/O139 NCTC 4711			+
V.alginolyticus NCTC 10675			-
V.vulnificus NCTC 11067			+
Salmonella typhimurium ATCC 14028			-
E.coli ATCC 25922			-
A. hydrophila ATCC 35654			-
P. aeruginosa ATCC 27853			-

Predatory activity of HBXCO1 towards *Vibrio* and non *Vibrio* strains

Methods

One 72 h laboratory-scale *V. parahaemolyticus* decontamination experiment by HBXCO1 was performed, with 10^5 PFU per ml/10^6CFU per ml predator/prey ratio, at 17°C and 3% salinity in an experimental aquarium with Artificial Sea Water (ASW) on mussels (*Mytilus galloprovincialis*) from an authorized harvesting area of the Central Adriatic Sea (Italy). At 24, 48, 72 after contamination, predator and prey were enumerated in microcosm test (with predator and prey) and control (with prey alone). Double layer agar plating technique was used to enumerate HBXCO1 from ASW of test microcosm by combining 1 ml of host (at an OD600 of 0.20) and 7.5 ml of undiluted and diluted ASW to 7.5 ml of molten (48°C) Pp 20 agar in tubes. The tubes were poured on top of the existing bottom layer (Ottaviani et al., 2018). Transmission electron microscopy and 16S rRNA analysis was used to identify *Halobacteriovorax.* Counts of *V. parahaemolyticus* were performed on test and control microcosms, by pour plate technique. Briefly, 10 g of body and intervalvular liquid, obtained from 10 mussels, were 1:10 diluted in physiological saline solution (ISO 6887-3 2017), homogenized, and serially diluted in the same buffer. Then, 10 ml of each dilution were inoculated onto three plates of TCBS agar (3, 3 and 4 ml) and these incubated at 37°C for 24 h. Analogous test (with predator and prey) and control (with prey alone) microcosms, without mussels, were prepared and analysed.For predatory efficiency of HBXOC1, three-day enrichments of HBXOC1 (approximately 1× 106 PFU ml^{-1}) were filtered through a 0.45- m-pore-size Millex HV syringe filter (Millipore Corp., Billerica, MA) to remove primary prey, allowing passage of the smaller HBXOC1. Prey specificity and predator efficiency of HBXOC1 were determined by monitoring its abilities to form clear lytic halos with double layer agar plating technique on a lawn of *Vibrio* and non-*Vibrio* preys.



The population dynamics of HBXCO1 and *V. parahaemolyticus* (Vp) in test (with HBXCO1) and control (without HBXCO1) microcosms with mussels (A) and without mussels (B) ♦ Vp control; ● Vp test; ▲ HBXCO1 control; ■ HBXCO1 test

Results and Discussion

In the test microcosm with mussels, the concentration of *V. parahaemolyticus* remained constantly around 5 log for the whole test period. In the control microcosm with mussels, between 0 and 6 h *V. parahaemolyticus* concentration remained constant, from 6 to 24 h increased by about 2 log, from 10^5 to 10^7 CFU per ml, from 24 to 48 h it leveled off and finally, from 48 to 72 h it decreased by about 1 log from 107 to 106 CFU per ml. At 24, 48, 72 h *V. parahaemolyticus* concentrations were significantly lower in the test than in the control microcosm, with the maximum difference of 2.2 log at 24 h. In the test and control microcosms without mussels, the trends of *V. parahaemolyticus* were comparable to those obtained in the respective microcosms with mussels, suggesting that the mollusk matrix does not affect the efficacy of HBXCO1 HBXOC1 shows prey specificity for the *Vibrio* genus and high predatory efficiency towards a wide range of pathogenic strains. Results of this study indicates HBXCO1 as a potential candidate in developing depuration strategies to improve conventional control of *V. parahaemolyticus* and other pathogenic vibrios in bivalves.

Bibliografia

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This work was supported by Italian Ministry of Health (grant RC 005 2016)