MOLECULAR SURVEY OF DISEASES IN MOLLUSCS CULTIVATED ALONG THE EASTERN ADRIATIC COAST

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Summary

Croatian mollusk industry is based on cultivation of European flat oyster (*Ostrea edulis*) and Mediterranean mussel (*Mytilus galloprovincialis*). Listed mollusks diseases may have very serious influence on this aquaculture activity. That is the reason why the National surveillance programme was put in place in 2000. During last ten years samples of oysters and mussels were taken regularly in all production area with aim to determine their health status with regard to listed parasites *Bonamia ostreae* and *Marteilia refringens*. Since the first record of *Marteilia* sp. in mussels in 2000. with a prevalence of 5%, the parasite is now enzootic with varying prevalence among 0 to 10% in different production areas. Parasite prevalence is independent of cultivation density. Although both species are cultivated in the same area oysters remains free of both parasites *B. ostrea* and *M. refringens.* In the diagnostic work cytological and histological methods were compared with molecular techniques and resulted with almost identical results. Infection of mussels by *Marteilia* spp. was confirmed by PCR. (ITS-1) region from *Marteilia* spp. parasitizing Mediterranean mussels was characterized by RFLP with endonuclease HhIal and sequenced. Phylogenetic affinity of the sequences was determined.

Introduction

Croatian mollusk industry is based on cultivation of European flat oyster (*Ostrea edulis*) and Mediterranean mussel (*Mytilus galloprovincialis*). Epizootics of two protozoans, *Marteilia refringens* (Grizel et al. 1974) and *Bonamia ostreae* (Pichot et al. 1980) devastated the production of the flat oyster *O. edulis* in Europe and therefore diseases caused by both parasites are notifiable for OIE (2010) and European Community (2006/88/EC). Likewise Croatian MAFRD initiated National surveillance programme in 2000. At the early beginning of the conduction of the programme, *Marteilia* sp. was detected in mussels (Zrncic et al. 2001) with prevalence varying from 0 do 5% in all production areas. In this study results of surveillance by means of traditional methods; cytology and histology were compared by molecular techniques during last two years.

Material and methods:

Samples analyzed in this study are shown in Table 1.

Table 1. Samples of *O. edulis* and *M. galloprovincialis* analysed 2010.

SAMPLING POINTS		DIAGNOSTIC TOOL								
		Detection of **Bonamiosis**			Detection of **Marteiliosis**					
		Heart imprints	HISTO	PCR	imprints of dig.gland	HISTO	PCR	imprints of dig.gland	HISTO	PCR
ZONE	POINT	OYSTERS			OYSTERS			MUSSELS		
I ISTRA	Limski kanal				0/50#	0/10	0/50	0/30	0/10	0/30
	Vabriga							0/30	0/10	0/30
	Porto Budava							2/30	0/10	2/30
	Savudrijska vala							5/30	2/10	5/30
	Raški zaljev							3/30	2/10	3/30
II MIDDLE ADRIATIC	Strmica							0/30	0/10	0/30
	Kanal sv.Ante							0/30	0/10	0/30
	Šibenik M1							0/30	0/10	0/30
	Pirovac							0/30	0/10	0/30
	Novigradsko more							0/30	0/10	0/30
	Modrić							0/30	0/10	0/30
	Uvala Stara Povljana	0/50	0/10	0/50	0/50	0/10	0/50			
	Dinjiška							0/30	0/10	0/30
III MALI STON BAY	Bistrina	0/50	0/10	0/50	0/50	0/10	0/50	0/30	0/10	0/30
	Sutvid	0/50	0/10	0/50	0/50	0/10	0/50	0/30	0/10	0/30
	Bjejevica	0/50	0/10	0/50	0/50	0/10	0/50	0/30	0/10	0/30
	Mali Ston	0/50	0/10	0/50	0/50	0/10	0/50	0/30	0/10	0/30
	Brijesta	0/50	0/10	0/50	0/50	0/10	0/50	0/30	0/10	0/30
	Sobra							0/30	0/10	0/30

number of positive/number of analysed

Tissue (heart or digestive gland) imprints were prepared and stained using Giemsa-modified staining method (Hemacolor Kit, Merck). Visceral masses of the oysters and mussels intended for histological examination were cut along the sagittal plane and placed in Davidson's fixative. The sections were subsequently treated by conventional histological procedures. Sections were cut 2 µm thick and stained with hematoxylin and eosin.

For *Bonamia* spp. DNA was extracted from pieces of gills. PCR based on amplification of partial sequence of small subunit ribosomal RNA with primers BO and BOAS was used in current survey. DNA for *M. refringens* was extracted from digestive gland tissues using the DNA blood and tissue kit (Qiagen). Amplification of a fragment (around 410 bp) of the internal transcribed spacer (ITS-1) was obtained by using the forward M2A and reverse M3AS primers. Amplified samples were purified using an ExoSAP-IT® (USB) and sequenced in both directions by the commercial company (Macrogen Inc.). Sequences were assembled using the SeqMan II software.

Fig.1. Map of Croatia with sampling points (note: red symbols highlight points positive for *M. refringens*).

Results and discussion

Both heart imprints and imprints of digestive glands of oysters were negative for *B. ostrea* and *M. refringens*. The histological findings of oysters' tissues did not reveal any of mentioned parasites. Described PCR analyses of oyster's tissues (gills and digestive glands) were negative for both parasites.

But developmental stages of *M. refringens* were noticed in digestive glands imprints (Fig.2) as well as in histological slides (Fig. 3) of mussels sampled on three different sites (Table 1., Fig.1).

All sequences were identical to partial sequence of *M. refringens* type M (Acc. No. AY324556) from Spain and 99% to isolate from *M. refringens* detected in *M. galloprovincialis* from Tyrrhenian Sea. From the results of the study it is obvious that the prevalence of *M. refringens* type M in musses is very low. Sequences of isolates from all infected sites were 100% identical and almost identical (99%) to isolates or *M. refringens* type M described by Novoa et al. (2005) and isolate from mussels in Tyrrhenian Sea (unpublished data). Further research should be forwarded to the impact of the parasite to mussel industry in all European areas.

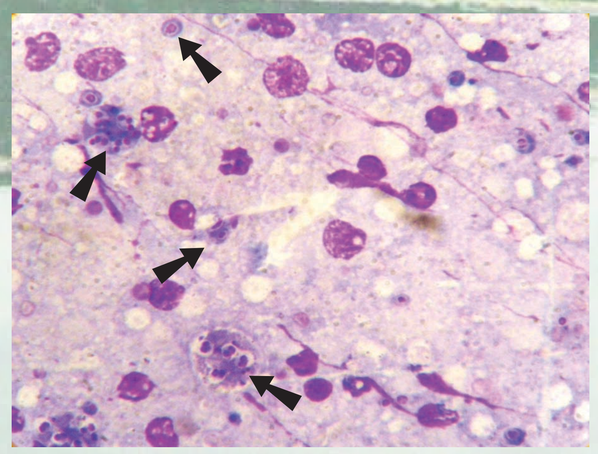

Fig. 2. Mediterranean mussel's digestive gland imprint showing young stages and sporangia of *M. refringens*.

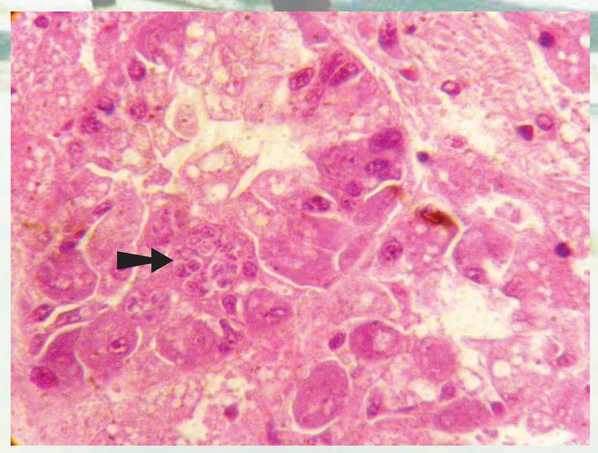

Fig. 3. Sporulating stages of *M. refringens* in digestive gland tubule of mussel.

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