Available online at www.sciencedirect.com
ScienceDirect
Journal of Invertebrate Pathology 98 (2008) 190–197
Journal of INVERTEBRATE PATHOLOGY
www.elsevier.com/locate/yjipa

Assessment of haemic neoplasia in different soft shell clam *Mya arenaria* populations from eastern Canada by flow cytometry

Maryse Delaporte [a,d,*], Stéphanie Synard [a], Julie Pariseau [a,b], Patricia McKenna [a], Réjean Tremblay [b], Jeffery Davidson [c], Franck C.J. Berthe [a,e]

[a] *University of Prince Edward Island, Atlantic Veterinary College, Department of Pathology and Microbiology, Charlottetown, C1A 4P3, Prince Edward Island, Canada*
[b] *Institut des Sciences de la mer, Université du Québec à Rimouski, Rimouski, G5L3A1, Que., Canada*
[c] *University of Prince Edward Island, Atlantic Veterinary College, Department of Health Management, Charlottetown, C1A 4P3, Prince Edward Island, Canada*
[d] *Université Louis Pasteur, Institut de Virologie, 3 rue Koeberlé, 67000 Strasbourg, France*
[e] *Animal Health and Welfare Unit, European Food Safety Agency (EFSA), Largo N. Palli 5/A, I-43100 Parma, Italy*

Received 8 October 2007; accepted 20 December 2007
Available online 28 December 2007

Abstract

Diagnosis of haemic neoplasia (HN) in the soft shell clam, *Mya arenaria*, is often achieved by hematocytology and histology. Since neoplastic cells display tetraploid DNA contents, haemocyte cell cycle analysis was developed for use as a diagnosis tool. The aim of this study was to assess the application of a flow cytometry procedure of cell cycle analysis established for the common cockle, to clams and to evaluate different thresholds of value for the percentage of tetraploid cells for establishing HN disease status of individual clams and clam populations. HN status of six clam populations from eastern Canada was determined. Results of the present study demonstrate a flow cytometry procedure to be useful for HN diagnosis in clams. Individual clams were considered to be affected by HN when presenting at least 20% of haemocytes in S–4N phase; and negative when presenting less that 5% of haemocytes in S–4N phase. As discussed in this paper, intermediate cases represent uncertain diagnoses including either false-negative or false-positive clams, which are difficult to discriminate. At a population level, an additional threshold of 15% for the mean intensity of the disease is proposed, which means having in the population several individual clams presenting more than 20% of their haemocytes in S–4N phase. Based on these thresholds of value, only one population was considered as free of HN disease, and one population was unequivocally affected by HN. For the four other clam populations, further investigations are needed toward development and use of specific and objective biomarkers of HN.

Keywords: Haemic neoplasia; Population assessment; *Mya arenaria*; Flow cytometry; Cell cycle; Threshold; Cut-off values

1. Introduction

Disseminated neoplasias (DN) have been reported in a variety of bivalve molluscs around the world since the initial description by Farley (1969a, 1969b). The condition has been most intensively studied in the soft shell clam, *Mya arenaria*, in which the disease is referred as haemic neoplasia (HN), and is progressive and fatal in most cases. Three comprehensive papers consider the published literature in detail and critically reviews subjects including diagnosis methodology and detection (Peters, 1988; Elston et al., 1992; Barber, 2004).

HN diagnosis is often based on hematocytology, coupled or not with histological tissue examination (Brousseau, 1987; Brousseau and Baglivo, 1991a; da Silva et al., 2005; Dungan et al., 2002; Elston et al., 1988; McGladdery et al., 2001; Villalba et al., 2001). Haemolymph-screening

* Corresponding author. Address: Université Louis Pasteur, Institut de Virologie, 3 rue Koeberlé, 67000 Strasbourg, France. Fax: +33 3 90 24 37 24.
E-mail address: maryse_delaporte@hotmail.com (M. Delaporte).

0022-2011/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.jip.2007.12.005

techniques enable distinction of neoplastic cells based on their morphology. According to the literature, neoplastic cells have a large nucleus and a high nucleus:cytoplasm ratio. The intensity of HN is generally rated as the percentage of neoplastic cells in the total number of haemocytes examined per slide (Farley et al., 1986), and classified in 3 to 5 different stages, depending on authors' classification criteria (Brousseau and Baglivo, 1991b; Farley et al., 1986; Leavitt et al., 1990; McGladdery et al., 2001). Brousseau and Baglivo (1991b) separated clams into three groups relative to the intensity of the disease: non-affected, low severity (1–50% neoplastic cells) and high severity HN (>51% neoplastic cells) while McGladdery et al. (2001) ranged intensity of HN disease as light (1–10%), moderate (11–50%) and heavy (51–100% neoplastic cells).

Complementarily, histopathology provides information on disease pathology within tissues and main organs. Mix (1983) assigned 4 stages of disease intensity for mussels, depending on infiltration rate of neoplastic cells throughout the body based on histological examination. Although hematocytology and histopathology are broadly used diagnostic methods, they present at least three disadvantages: the limited number of cells analysed per slide, subjectivity, and high labour cost for processing and analysing samples.

In the 1990s, flow cytometry methods (FCM) became increasingly common for studying haemocyte types and functions in bivalve mollusc species (see Ashton-Alcox et al., 2000 for review) and also ploidy changes in the HN disease (da Silva et al., 2005; Elston et al., 1990; Harper et al., 1994; Moore et al., 1991; Reno et al., 1994). Analysis of cell cycles by FCM has allowed establishing that relative amounts of DNA vary between normal and neoplastic cells, and that these differences are not the same among species. It has been demonstrated that the blue mussel, *Mytilus edulis*, affected by DN contained tetraploid and pentaploid cells with 2.03 to 2.64 times more DNA than normal diploid haemocytes (Elston et al., 1990). In the soft shell clam *M. arenaria*, neoplastic haemocytes had 1.25 to 2.05 time more DNA than normal diploid haemocytes (Reno et al., 1994). More, recently, da Silva et al. (2005) established that the common cockle *Cerastoderma edule* presented hypodiploid, hyperdiploid, triploid, and pentaploid neoplastic haemocytes. FCM has proven to be a more powerful and accurate diagnosis tool than traditional diagnosis methodologies, since it allows screening large numbers of cells in few seconds, and permits simultaneous multi-parametric data acquisitions per cell (size, granularity, and fluorescence intensity).

Although DN status of *M. edulis* and *C. edule* may be obvious when considering animals with pentaploid cells, assessment of HN status of *M. arenaria* clams appears to be more challenging, especially for lightly affected clams. Indeed, distinction of lightly affected clams exhibiting few abnormal tetraploid cells, from negative clams with potentially normal tetraploid circulating cells in mitotic process had never been discussed. This shortcoming may result from the fact that the hematopoiesis process remains unknown in molluscs, and that many authors have worked with heavily affected clams.

The aim of this study was to determine thresholds of value for the percentage of tetraploid cells obtained by FCM for establishing HN disease status of individual clams, and populations. HN assessments of clams from 6 different *M. arenaria* populations from eastern Canada were performed according to the FCM procedure of da Silva et al. (2005) for *C. edule*.

2. Material and methods

2.1. Clam collection

Soft shell clams *M. arenaria* were collected at low tide from the following 6 populations of eastern Canada (60 clams per population), and brought to the Atlantic Veterinary College (Fig. 1): Anse St-Étienne Bay (QC), Métis Bay (QC), Kouchibougouac National Park (NB), Havre-aux-Maisons lagoon (Magdalen Islands, QC), North River (PE), and Barasway Bay (NL). Upon arrival at the laboratory, clam populations were maintained separately in tanks with static seawater at 18 °C and salinity 28‰, for two days to one week before flow cytometry analysis.

2.2. Haemolymph preparation and flow cytometry analysis

Haemolymph was withdrawn from each clam's anterior adductor muscle using a 3-ml syringe fitted with a 25-gauge needle. A volume of 500 µl of haemolymph was fixed in absolute ethanol (1:5), and treated according to da Silva et al. (2005). Briefly, after centrifugation at 400*g* for 10 min, cells were re-suspended and re-hydrated for 30 min in Saline Phosphate Buffer (PBS, 0.01 M), twice washed with saline PBS (0.01 M), and filtered through an 80-µm nylon mesh while transferring to a flow cytometer tube. Samples were then treated with DNase-free RNase A (Sigma R4875, 50 µg ml^{-1}) and stained with propidium iodide (PI, Sigma, P4170, 50 µg ml^{-1}) for 45 min. PI fluorescence, which is related to the DNA content of each cell, was detected on the FL2 detector (orange light, at 550–600 nm) of a FACSCalibur flow cytometer (Becton–Dickinson). For each sample, 10,000 particles were counts at low flow rate (15 µl min^{-1}).

On cytograms plotting the width and area of PI fluorescence signals, regions (or gates) related to normal diploid cells (G0/G1), phase S cells, and tetraploid (G2/M) cells were drawn, and considered to represent the mean fluorescence of each category of cell types (Fig. 2a). PI fluorescence intensities of single cells were also plotted on a FL2-area histogram on which specific markers were placed to estimate the percentage of normal cells, phase S, and tetraploid cells in the analysed cell population (Fig. 2b). Results of haemocyte cell cycle analysis are presented on histogram-area, 3-axis graphs, on which the *X*-axis is divided into discrete categories representing increasing