Faculty of Resource Science and Technology

APPLICATION OF WHOLE-CELL FLUORESCENT *IN SITU* HYBRIDIZATION (FISH) FOR MOLECULAR DETECTION OF TOXIC *ALEXANDRIUM* SPECIES

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Application of whole-cell fluorescent *in situ* hybridization (FISH) of molecular detection of toxic *Alexandrium* species

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Declaration

No portion of the work referred to this report has been submitted in support of an application for another degree of qualification of this or any other university of institution of higher learning.

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List of abbreviations

APC	Apical pore complex
BLAST	Basic Local Alignment Search Tool
CPD	Critical Point Dried
FISH	Fluorescence *in situ* Hybridization
FITC	Fluorescein-5-isothiocyanate
HAB/s	Harmful Algal Bloom
LM	Light Microscopy
MP	Maximum Parsimony
PAUP	Phylogenetic Analysis Using Parsimony
PSP	Paralytic Shellfish Poisoning
SEM	Scanning Electron Microscopy
STX	Saxitoxin
TBR	Tree Bisection-reconnection

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Application of whole-cell fluorescent *in situ* hybridization (FISH) for molecular detection of toxic *Alexandrium* species

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ABSTRACT

Paralytic shellfish poisoning is a type of seafood poisoning due to contamination of a group of neurotoxin, collectively known as saxitoxin, in filter feeding mollusks. Monitoring of PSP producing organism especially from the genus of *Alexandrium* is very challenging as identification of toxic species is only possible based on plate tabulation. In this study, oligonucleotide probes using whole cell fluorescence *in situ* hybridization (FISH) to detect toxic *A. tamiyavanichii* and *A. minutum* were developed. *In silico* probe design for the 2 species was carried out using sequences database of *Alexandrium* species found in Malaysia. Probe specificity was assessed based on GC content, melting temperature, Gibbs free energy change and E value. Potential species-specific probes for *A. minutum and A. tamiyavanichii* were designated as [L-S-Amin-680-(*A. minutum*)-A-24] and [L-S-Atam-286-(*A. tamiyavanichii*)-A-23]. Specific probe for *A. tamiyavanichii*, TAMIS1 was synthesized and tested against *A. tamiyavanichii* and all other related species. Probe uniC and uniR were applied as positive and negative control. Our result show high specificity of TAMIS1 to *A. tamiyavanichii* with no cross-reactivity to four other *Alexandrium* (*A. minutum, A. leei, A. tamarense* and *A.affine*). FISH condition applied in this study with hybridization temperature, 35 ℃ and formamide 40 % work well with no unspecific binding observed. The probe TAMIS1 should be adopted in national HAB monitoring program in order to increase the efficiency in detection of *A. tamiyavanichii*.

Key words: *Alexandrium minutum*, *A. tamiyavanichii*, FISH, oligonucleotide probes, TAMIS1 probe

ABSTRAK

Keracunan paralitik kerang-kerangan adalah jenis keracunan makanan laut akibat pencemaran toksin saraf, yang dikenali sebagai saxitoksin, dalam moluska pemakan saringan. Pemantauan organisma menghasilkan PSP terutama dari genus Alexandrium adalah mencabar kerana klasifikasi spesies beracun ini hanya berdasarkan susunan kepingan permukaan sel. Dalam kajian ini, pendekatan prob oligonukleotida dengan seluruh sel hybridisasi pendaran in situ (FISH) dibangunkan untuk mengesan sel beracun A. tamiyavanichii dan A. minutum . Prob in silico direka untuk kedua-dua spesies dilakukan dengan menggunakan pengkalan data jujukan gen spesies Alexandrium yamg ditemui di Malaysia. Kesesuaian prob dianalisa berdasarkan kandungan GC, suhu lebuh, perubahan tenaga bebas Gibbs dan E-nilai. Prob spesies-spesifik yang berpotensi untuk A. minutum dan A. tamiyavanichii dinamakan sebagai [L-S-Amin-680-(A. minutum)-A-24] dan [L-S-Atam-286-(A. tamiyavanichii)-A-23]. Prob A. tamiyavanichii TAMIS1 yang *disintesis dan telah diuji terhadap A. tamiyavanichii dan semua spesies berkaitan yang lain. Prob uniC dan uniR telah digunakan sebagai kawalan positif dan negatif. Keputusan kami menunjukkan spesifisiti yang tinggi TAMIS1 dalam pengesanan A. tamiyavanichii tanpa reaksi silang terhadap empat Alexandrium yang lain (A. minutum, A. leei, A. tamarense dan A. affine). Keadaan FISH yang digunapakai dalam kajian ini dengan suhu hibridisasi, 35 ℃ and formamide 40 % berfungsi dengan baik tanpa pengikatan tidak spesifik yang dicerap. Pendekatan molekul harus diserap dalam program pemantauan HAB kebangsaan demi meningkatkan kecekapan dalam pengesanan A. tamiyavanichii.*

Kata kunci: Alexandrium minutum, A. tamiyavanichii, FISH, prob oligonukleotida, prob TAMIS1

1.0 Introduction

Marine dinoflagellates are one of the most important phytoplankton besides diatoms in marine ecosystem. They are important microscopic component of the marine food web. However, they also bring negative effects that cause economic losses to aquaculture and fisheries industries, as well as environmental deterioration and human health problems. Dinoflagellates have attracted attention from the public due to the production of neurotoxins that causes shellfish poisoning. Among the shellfish poisoning incidents, paralytic shellfish poisoning (PSP) is the most pivotal incidence due to its casualty caused by the toxins, saxitoxins (STXs). In dinoflagellates, there are three species that produce such a potent neurotoxins, they are *Pyrodinium bahamense*, some species of *Alexandrium* and *Gymnodinium catenatum* (Kodama, 2010). *A. minutum* and *A. tamiyavaichii* in this study is the PSP toxin producers that have been found in the Malaysian waters (Usup *et al.*, 2002; Lim *et al.*, 2005).

Morphological characteristics of *Alexandrium* are based on key diagnostic features such as overall cell shape and Kofoidan theca plate tabulation. Identification of *Alexandrium* species was based primarily on the morphology of the following plates: posterior sulcal (S.p.), second antapical (2""), first apical (1'), anterior sulcal (S.a.), third apical (3'), sixth precingular (6") and the apical pore complex (APC). Other features used in identification were the presence and location of the ventral pore as well as the anterior and posterior attachment pores (Balech, 1995; Fukuyo, 2001; Leaw *et al.*, 2005).

Under light microscopic observation, cells of *Alexandrium* are difficult to distinguish since population of *Alexandrium* species may coincide in the nature, whereas toxic properties are very divergent (Cembella *et al.*, 1998; John *et al.*, 2003). Furthermore, the identification of *Alexandrium* up to species level is time consuming due to their

complexity in the morphological features (Sako *et al.*, 2004). Thus expertise is needed in precise species identification. More reliable and rapid identification methods and tools are needed. As such molecular identification of toxic *Alexandrium* by whole cell fluorescence *in situ* hybridization (FISH) had been developed (Kim *et al.*, 2005). There are several probe types that had been applied in FISH methods.

FISH method is very useful for evaluation of molecular phylogenetic identity, number and spatial arrangements of microorganisms in environmental setting (Amann *et al.*, 1995). It provides qualitative and semi-quantitative results in detecting and quantifying the target cells.

This study aimed to apply a molecular approach to rapidly detect the toxic *Alexandrium* species from Malaysia. The specific objectives are as below:

1. To develop species-specific oligonucleotide probes for rapid detection of toxic *Alexandrium* species,
2. To apply whole cell fluorescence *in situ* hybridization (FISH) in detecting toxic *A. tamiyavanichii*,
3. To test the specificity of species-specific probe towards *A. tamiyavanichii* cells.

2.0 Literature Review

2.1 Harmful algal bloom (HABs)

Algal blooms are common occurrence in marine and freshwater ecosystem. HABs occurred when certain types of microscopic algae such as dinoflagellates grow quickly in water, forming visible patches that may bring adverse effect to human, environment, plants or animals' health including massive fish mortalities, seafood poisoning in human and biofouling of beaches and fishing gear (John *et al.*, 2003). During a HAB event, algal toxins can accumulate in predators and organisms through food web (Backer and Mcgillicuddy, 2006).

The first report of HABs event occurred in Malaysian water was in 1976 when toxic dinoflagellate *Pyrodinium bahamense* var. *compressum* bloomed in the west coast of Sabah (Roy, 1977). Several people were poisoned during this event. Blooms of this species have continued to occur almost annually in the state (Usup and Azanza, 1998).

HABs can deplete the oxygen and block the sunlight that needed by plants for photosynthesis and some HAB- causing algae release toxins that bring harm to animals and humans. HABs appear to be increasing along coastlines and surface waters. The probably best known human health effects that caused by HAB related organisms are shellfish poisonings. There are several types of shellfish poisoning such as amnesic, diarrhetic, neurotoxic, ciguatera and paralytic shellfish poisoning.

2.2 History of Paralytic Shellfish Poisoning (PSP)

PSP toxins are produced predominantly by marine dinoflagellates which include *Alexandrium* species. The toxins collectively referred as saxitoxin (STX) which is blockers of voltage-gate sodium channels found on nerve cells and smooth muscles (Shimizu, 1994) (Figure 2.1). Doucette *et al.*, (1997) figured out that STX will bind to the voltage dependent sodium channel and inhibits channel open. Action of these toxins inhibits depolarization of nerves and smooth muscles resulting in paralysis and death due to respiratory failure (Usup *et al.*, 2002). This toxin will cause the victim tingling sensations, headaches, fever, rash, dizziness, gastrointestinal illness, muscular paralysis, pronounced respiratory difficulty and choking sensation. However, about one to four milligram of the sensation of the STX will become lethal and caused respiratory paralysis and loss motor control (Evans, 1972). Saxitoxins are passed on to marine life that feed on microalgae. Generally, saxitoxins are soluble in water and can withstand high temperature. It is stable at acidic conditions and will degrade in alkaline conditions (Kodama, 2010).

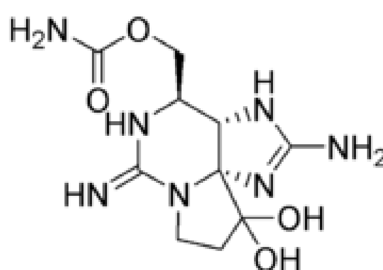

Figure 2.1: Chemical structure of saxitoxin (Ostlund *et al.*, 1974)

In Malaysia at least four PSP toxin producers are currently known viz. *Pyrodnium bahamense* var. *compressum* in Sabah, *Alexandrium tamiyavanichii* in Sebatu Melaka, *A. minutum* and *A. lusitanicum* in Tumpat Kelantan (Usup *et al.*, 2002). The primary vector for PSP toxin is bivalve mollusks. Victims that suffer from PSP toxin will have symptoms such as neurological, heart arrest in most severe cases after 24 hours and closure of mussel

beds. The first PSP incident that happens in east coast of Peninsular Malaysia is caused by *A. minutum* where by six person were poisoned and included one fatally was reported after consumption of contaminated benthic clam *Polymesoda* sp. Collected from a coastal lagoon in Tumpat, Kelantan (Usup *et al.*, 2002).

2.3 The genus *Alexandrium*

There are 33 species of *Alexadrium* currently described (Leaw *et al.*, 2009). The species were classified based on their morphological features such as cell form, shape of apical pore plate, the presence of a ventral pore on 1' apical plate and the position of posterior and attachment pore (Balech, 1995) (Figure 2.2). Some species of *Alexandrium* were found to be toxic and some are not. The *Alexandrium* species which have the ability to produce STX included *A. tamiyavanichii*, *A. caternella*, *A. fundyense*, *A. minutum* and *A. tamarense*. Below is the taxonomy of *Alexandrium* according to Halim (1960).

Kingdom : Protoctista

Phylum : Dinoflagellata

Subphylum : Pyrrhophyta

Class : Dinophyceae

Order : Gonyaulacales

Family : Goniodomaceae

Genus : *Alexandrium*

The morphological differences among *Alexandrium* species were minute; the differences can be observed in the shapes and arrangement of the thecal plates. However this can only be investigated under high magnification of microscope with the aids of

fluorescence optic system. Thus it is very difficult to discriminate the species in this genus. This difficulty has rise the doubt of the validity of identifications based solely on the morphological characteristics. Therefore, probes design and FISH technique is developed.

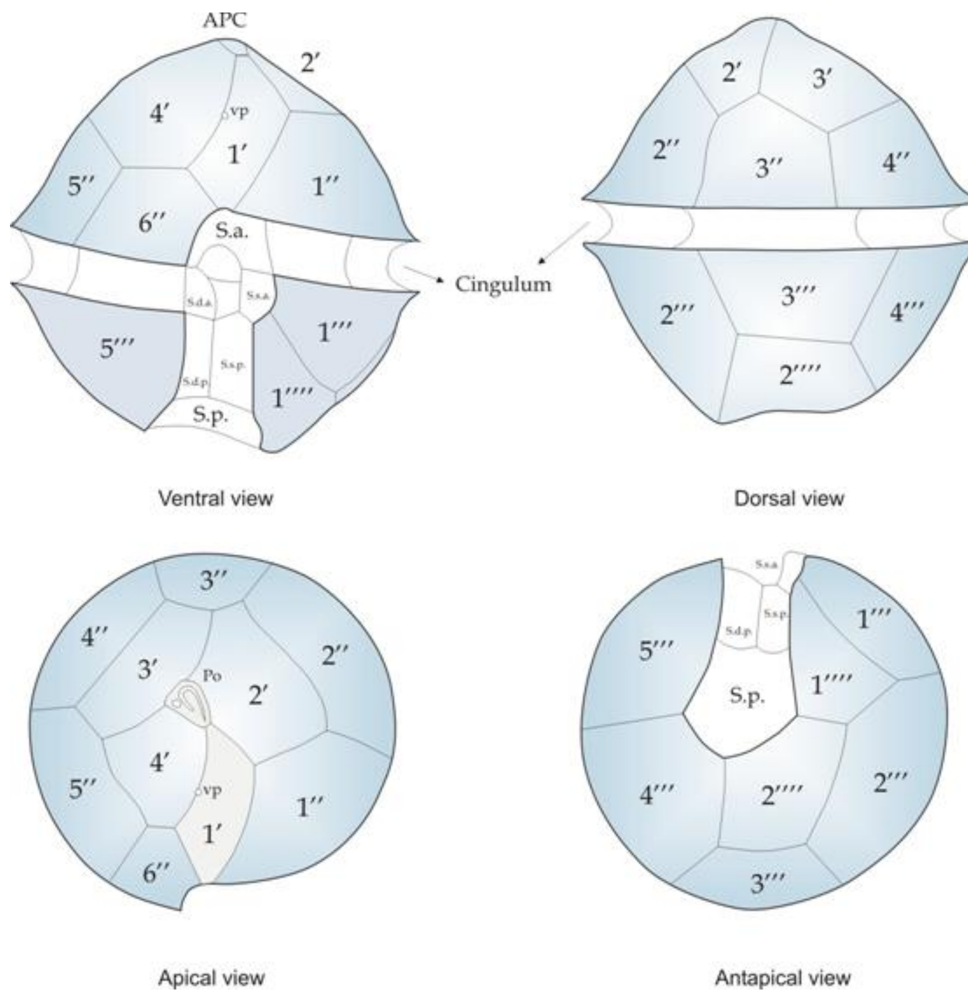

Figure 2.2: Theca plate tabulation of *Alexandrium* species showing the ventral, dorsal, apical and antapical views. Apical plates are represented as ('), precingular plate as (''), postcingular plates (''') and antapical plates ('''') (adopted from Balech, 1995)

2.4 Fluorescence *in situ* hybridization (FISH)

FISH is a method whereby a fluorescently probe designed to recognize a specific sequence of a particular organism is hybridized inside intact cells and cell containing a complex of probes and the specific sequence are detected by using epifluorescence microscopy. (Hosoi-Tanabe and Sako, 2005). The first application of fluorescent *in situ* detection came in 1980, when RNA that was directly labeled on the 5' end with fluorophore was used as a probe for specific DNA sequences (Amann *et al.*, 1995). This technique is very beneficial that can be used to evaluate the phylogenetic identity, number and spatial arrangements of microorganisms in environmental setting (Amann *et al*., 1995).

FISH method has been successfully applied to some HABs species that reported which showed that FISH method is a powerful tool to detect specific species collected from cultured and natural samples. FISH has been applied in discriminating the toxigenic *A. tamarense* and *A. ostenfeldii* in co-occurring natural populations from Scottish coastal water. (John *et al*., 2003).

2.5 The ribosomal RNA genes precursor

In the past decade, the sequences of the rRNA gene family, 5.8S rDNA, 28S rDNA and the internal transcribed spacers (ITSs) region (Figure 2.3) have been used to determine several *Alexandrium* species (Scholin *et al.*, 1994; Adachi *et al.*, 1996a). The 28S rDNA sequences of some *Alexandrium* species indicated that D1 and D2 region could provide a species-specific genetic marker for the genus *Alexandrium* which reflects taxonomic classification based on phylogenetic inference of the genus (Scholin *et al.*, 1994; Scholin and Anderson, 1996). Furthermore, the region included interspecific and intraspecific taxonomic markers (Adachi *et al.*, 1996a). These genetic markers thus have been used as the basic detection of

Alexandrim species by fluorescence *in situ* hybridization (FISH) (Anderson, 1995; Miller and Scholin, 1996).

Figure 2.3: The single repeat of ribosomal RNA gene precursor (adopted from Cooke and Duncan, 1997)

2.6 Ribosomal RNA targeted oligonucleotide probes

More than 20 years ago, the development of rRNA gene targeted oligonucleotide probes by Stahl *et al.* (1988) have become an important milestone for the detection of microorganisms involved in biotechnological process. Ribosomal RNAs (rRNAs) gene act as an excellent target molecules because it is presence in all organism, high natural concentration and high information content that provide signature nucleotide content stretch for most phylogenetic taxa (Lipski *et al*., 2001).

A good probes design and careful further evaluation *in silico* plays an important role to ensure the sensitivity, specificity and consistency is satisfied (Kumar, *et al*., 2005). Sensitivity is required in designing probes in which self-complimentarily of probes and probes that intend to hybridize to themselves rather than to their targeted sequences need to avoid. Probes should also be specific for the intended gene or gene family and not complementary to other sequences so that cross-hybridization is inhibited. For consistency, the melting temperature for all probes should be within some small range so that they can

hybridize to their intended targets at the same temperature within an experiment (Feng and Tiller, 2007).

In optimization of the probe, long probes are preferred to be used instead of short probes because long probes yield better signal intensity than short probes. However, the signal intensity of short probes can be improved by addition of spacers or using higher probe concentration for spotting. The accurate gene expression measurement can be achieved with multiple probes per gene and fewer probes are needed if longer probes rather and vice versa. However, shorter oligonucleotide probes also work well in gene expression analysis if the probes are validated by experimental selection or if multiple probes per gene are used for expression measurement (Chou *et al*., 2004).

Selection of potentially “best” probes will be made on the following criteria: targeted sites located in the more conserved regions, target sites with maximum numbers of mismatches with non- targeted organisms and preference for centrally or near- centrally located mismatches (Simon *et al.*, 2000).

3.0 Materials and Methods

3.1 Sample collection and cell isolation

Plankton samples were collected from 2 different sampling sites in Sarawak by using 20 µm mesh size plankton net. The sampling sites are Samariang River and Santubong estuaries (Figure 3.1). The samples were brought back to the laboratory for further cell isolation as described below.

Figure 3.1: Kuching map showing the sampling locations, Santubong and Samariang.

Cells of interest were isolated by using micropipetting technique (Hoshaw & Rosowski, 1973). Desired algal cell for isolation was located using inverted microscope and washed by transferring them to drops of filtered water samples on the glass slides. The cell then was transferred into a well of tissue culture plate and placed in the culture cabinet. The clonal culture was examined daily until reach > 100 cells. Whole content will be transferred to a new culture tube of medium.

3.2 Algal cultures

Clonal culture of *Alexandrium* species used in this study was obtained from the UNIMAS phytoplankton culture collection in the Laboratory of Ecotoxicology (AmKB01, AmKB02, AmKB03, AmKB04, AmKB05, AmKB06, AcMS01, AaMS02, AtPA02 and AlMS02). The cultures were grown in SW II and ES-DK media at 25 ℃ under 12:12 h light: dark cycle.

Alexandrium minutum (AmKB01, AmKB02, AmKB03, AmKB04, AmKB05 and AmKB06) was cultured and maintained in SW II medium (Iwasaki, 1979) (Table 3.1). Filtered natural seawater was used as medium base. Salinity of the filtered seawater was adjusted to 15 PSU by diluting with ddH_2O. Medium was adjusted to pH 7.8 – 8.0 by adding 10 % HCl. *A. tamiyavanichii* (AcMS01), *A. affine* (AaMS02), *A. tamarense* (AtPA02) and *A. leei* (AlMS02) were maintained in ES medium (Kokinos and Anderson, 1995). A total of 9.3 mL ES-DK working stock and 1 mL f/2 vitamin stock were added to 1 L filtered seawater (Table 3.2). Salinity of filtered seawater was adjusted to 30 PSU by adding salt. The pH of medium was adjusted to pH 7.8-7.9 by adding 10 % HCl.

Table 3.1: SW II medium (Iwasaki, 1979)

Ingredients	Stock concentration	Volume added into 1L seawater (mL)	Final concentration
KNO_3	7.2×10^{-3} mol/L	1.0	7.2×10^{-4} mol/L
KH_2PO_4	3.31×10^{-4} mol/L	1.0	3.31×10^{-5} mol/L
Na_2-glycero.PO_4	3.31×10^{-4} mol/L	1.0	3.33×10^{-5} mol/L
Vitamin mix			
- B_{12} (cyanocobalamin)		1.0	4.43×10^{-10} mol/L
- Biotin			
- Thiamine-HCl			4.1×10^{-9} mol/L 3.0×10^{-7} mol/L
Fe-EDTA		1.0	1.19×10^{-6} mol/L
Tris-HCl (pH 7.8)		5.0	4.13×10^{-3} mol/L

Table 3.2: Final composition of ES-DK medium (Kokinos and Anderson, 1995)

ES-DK medium	g
Fe stock	
- $Fe(NH_4)_2(SO_4)_2.6H_2O$	0.351
- Na_2-EDTA	0.3765
P_2 stock	
- Na_2-EDTA	0.400
- $FeCl_3.6H_2O$	0.126
- H_3BO_3	0.456
- $MnSO_4.H_2O$	0.055
- $ZnSO_4.7H_2O$	0.0088
- $CoSO_4.7H_2O$	0.00192
ES working stock	
- $NaNO_3$	1.400
- Na-glycerophosphate	0.200
- P_2 stock	100 mL
- Fe stock	100 mL
f/2 vitamin stock	
- B_{12} (cyanocobalamin)	1 mL
- Biotin	10 mL
- Thiamine-HCl	200.0 mg
Seawater	1 L

The clean test tubes were soaked in 10 % HCl for overnight. The culture test tubes were rinsed with tap water and followed with distilled water. The culture test tubes were filled with about 25 mL of medium. The sterilization of culture test tubes were carried out by autoclaving at 121 ℃ and about 20 minutes. Media were left about 24 hours before

used to allow the CO_2 gases to diffuse into the liquid. Culturing was performed aseptically in a laminar flow hood.

3.3 Scanning Electron Microscopic (SEM) Observation

For cell fixation, sample was transferred into a centrifuge tube. Equal volume of 5 % glutaraldehyde fixative will be added and mixed. Then, the mixture was incubated at 4 ^{o}C for overnight. Cell suspension was centrifuged at 6,000 $\times g$ for 5 minutes and the fixative removed. Samples were washed three times using cacodylate buffered washing solution.

The fixed cells were transferred to a polycarbonate (PC) membrane by using vacuum manifold. The samples were dehydrated with graded EtOH series for 10-15 minutes at each concentration in vacuum manifold.

The substitution was carried out through graded bath (75:25, 50:50 and 25:75) of EtOH and amyl acetate for 15 minutes, before proceed immediately to critical point drying (CPD).

The specimen was loaded into CPD chamber with 100 % amyl acetate added gradually and filled the chamber with intermedium. The CPD chamber was pre-cooled and the chamber was filled with liquid CO_2. The media mixture was drained. The media mixture was purge and soak repeatedly 6-8 times until all solvent removed. Chamber was filled with LCO_2 subsequently heated above critical temperature. The pressure in chamber was reduced slowly until 0 psi. The dried specimens were removed.

The samples were mounted on an aluminum stub using a double stick carbon tape. Samples were coated with a very thin film of gold-palladium by sputter coater before observation under scanning electron microscope (SEM).