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One step immunochromatographic assay for the rapid detection of *Alexandrium minutum*

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Abstract:

Harmful algal blooms represent a major threat to marine production, and particularly to shellfish farming. Current methods for analyzing environmental samples are tedious and time consuming because they require taxonomists and animal experiments. New rapid detection methods, such as immunoassays, are sought for alerting purposes and for the study of algal ecodynamics in their natural environment. *Alexandrium minutum*, which causes paralytic shellfish poisoning, occurs with increasing frequency along European coasts. We have developed a one step immunochromatographic assay which is based on the principle of immunochromatographic analysis and involves the use of two distinct monoclonal antibodies directed against surface antigens of *A. minutum*. The primary specific antibody was conjugated with colloidal gold, and the secondary antibody (capture reagent) is immobilized on a strip of nitrocellulose membrane. We could demonstrate that whole algae are able to diffuse without restriction in the porous material. The assay time for this qualitative but highly specific assay was less than 15 min, suitable for rapid on-site testing.

Keywords: Antibody; *Alexandrium minutum*; Immunochromatographic assay (ICA); Rapid detection

1. Introduction

Harmful algal blooms (HABs), also commonly known as "red tides", are natural phenomena but their frequency, intensity, and geographic range have increased since the 1970s (Hallegraef 1993, 2003, VanDolah 2000). Furthermore, their economic impact is greater now than in the past, as a result of increasing consumption of seafood, growth of coastal populations and tourism industries. Different classes of toxins are produced by dinoflagellates; they accumulate in shellfish and are responsible for severe human syndromes. HABs are also harmful to the marine ecosystem as a whole, because algal toxins can sicken and kill many forms of aquatic organisms (Landsberg 2002). The genus *Alexandrium* is among the most harmful since species produce potent neurotoxins such as saxitoxins and gonyautoxins which are responsible for the so-called "paralytic shellfish poisoning". Monitoring of HAB species is tedious, and requires direct human expertise. The methods used for identification of algae are usually based on morphological studies under light microscopy. Unfortunately, algal morphology might change depending on environmental conditions and growth phases.

Recently, highly specific laboratory methods based on genetic information have been reported, such as fluorescence *in situ* hybridization (FISH) (Sako et al., 2004, Anderson 2005) or real-time PCR (Dyhrman et al.,2006). A method to detect *A. minutum* in a complex background using sandwich hybridization assay was also reported, but RNA isolation and sensitivity still need to be improved (Diercks et al., 2008). Antibodies represent a powerful tool in detection assays and the literature reveals many attempts to generate specific antibodies against harmful algae.

In a previous study (Gas et al., 2009), we developed a whole cell enzyme-linked immunosorbent assay (ELISA) based on highly specific monoclonal antibodies (mAbs) that recognize antigens at the surface of *A. minutum*. This assay required several incubation and washing steps, restricting its use d to laboratories or trained users. Taking advantage of this highly specific antibody, the current study aimed to develop a one step immunochromatographic assay (ICA) for the rapid qualitative detection of *A. minutum*. This assay is easy to use and is based on the principle that the antigen loaded into the sample area migrates on a nitrocellulose membrane strip. As a result of an immunoreaction, the algae are sandwiched between mAb-conjugated colloidal gold and another mAb immobilized on the assay strip. The result is determined by a visual line of red colored colloidal gold. This assay format has been widely developed for environmental applications. These test strips are used in water samples for chemical detection (Zhu et al., 2008) (Guo et al., 2009), and as drug metabolites sensors (Li et al., 2008). Toxins such as the brevetoxins in fishery product samples (Zhou et al., 2009) and staphylococcus enterotoxins B in contaminated food (Khreich et al., 2008) can be detected. Bacterial detection such as *Vibrio harveyi* has also been reported (Sithigorngul et al., 2007).
However, the detection of algal cells is a challenge because the size of algal cells exceeds 10 µm and surface antigens are of low abundance. We report here the first ICA for whole algal cell detection.

2. Materials and methods

Antibodies

Two rat monoclonal antibodies of *A. minutum* were selected for this study: AMI6 and AMI11. Their induction, cloning and production have been previously reported (Gas et al., 2009). They are both IgGs and were purified on a protein G HiTrap1® affinity column (GE Healthcare Life Sciences) prior to labelling or immobilization.
The labelling of mAb AMI6 with colloidal gold-conjugation was performed by British Biocell International (UK). It was conjugated on 40 nm gold colloid and stored in final buffer 2 mM borax, pH 8.2, 0.095% sodium azide at an optical density at 520 nm of around 10. Clustering was controlled by transmission electron microscopy: a hundred particles were counted showing a percentage of singlets higher than 85%, and cluster size below 10 particules/cluster.
The goat anti-rat IgG antibody was obtained from Tebu-bio.

Chemicals and ICA components

The sucrose, TRIS buffer, BSA and all other chemical were purchased from Sigma-Aldrich.
All membranes and supports for ICA were purchased from Microdevice LdT (MDI India):
- nitrocellulose (ref. CNPC-SS12-L2-H50) is a membrane laminate with nominal pore size of 15 µm.
- polyester sample pads (ref. GFB-R7L) exhibit high absorption capacity and do not bind proteins. Their size was 27 x 260 mm, 0.6 mm thickness, and already included buffers and detergents.
- absorbent pads -sink pads- (ref. AP 080). Size 27x260 mm, 0.8 mm thickness.
- conjugate release matrix glass fiber (ref. PT-R5) is a sturdy material which acts as a reservoir for the conjugate and transfers the particles quickly to the membrane. Its size was 70 x 260 mm.
- plastic cassettes (ref. Device 1) are 4 mm thick polystyrene cassettes.

Immobilisation of reagents

Colloidal gold-labelled mAb AMI6 was diluted (1:1, v/v) with 20 mM TRIS containing 10 % sucrose, 0.5% BSA. The conjugate pad was prepared by passive immobilization of labelled AMI6 onto the glass fiber with an Airjet (XYZ 3000) and then dried. Capture antibodies were dispensed directly onto the nitrocellulose using a Biojet XYZ 3000: AMI11 (0.5 mg/mL) as the test line (2 µL per 1 cm line), and the goat anti-rat IgG (1 mg/mL) as the control line (1 µL per 1 cm line).

Assembly of the kit

The one step strip for ICA is composed of three pads (sample, conjugate and absorbent pads) as described in (Fig.1). They were pasted onto a nitrocellulose membrane, backing on adhesive plastic containing the specific anti-*A. minutum* (AMI11) and the goat anti-rat (IgG) as control. The conjugated pad containing the gold labelled mAb (AMI6) was pasted overlapping the nitrocellulose membrane by 4 mm. The sample pad was also pasted overlapping the conjugate pad by 2 mm. The absorbent pad was pasted on the other side of the plate. The whole assembled plate was cut lengthways and divided into strips with a guillotine cutter (CM 4000) 4 x 60 mm. The strip was finally inserted into the cassette housing. Then 50 µl of the sample was loaded in the sample area S and the test could be read after 15 min.

Dinoflagellates strains and culture

The *A. minutum* AM89BM strain came from the IFREMER Centre de Brest collection. The inoculum was maintained for transport at mid-exponential growth in f/2 Guillard and Ryther medium (Guillard, 1975). The cultures were then grown axenically at 18 ± 1°C under cool-white fluorescence light, at a photon flux of 150 µE/m^2/s with 14:10 LD photoperiods. At the end of the exponential growth phase, cells were harvested by centrifugation (5000 g, 10 min) and could be kept frozen at -70°C as a stock solution for later use. We have previously shown (Gas et al., 2009) that results were similar for immunodetection of frozen and fresh cells. So, each sample used for the immunochromatographic assay was prepared from these frozen cells stock solutions, by dilution in sterile seawater. As the dinoflagellate contained thecal plates, the cellular lysates were prepared by disruption with a French press at 1kbar, then sonicated (pulse 5 s, stand by 5 s, for 2 min 30 s) at 4°C.

Fluorescence microscopy

Pellets of *A. minutum* cells were resuspended in seawater at approximately 10^6 cells/mL. 100 µl were loaded onto the sample pad and were visualized with fluorescence microscopy at the beginning and after total migration along the strip. The microscope used was a NIKON Eclipse TE 2000 E objective x200 and visualized the fluorescence signal after excitation at 460 nm-510 nm filter and with 250 ms acquisition time parameter.

3. Results and discussion

3.1 Behaviour of *Alexandrium minutum* (whole and lyzed) cells in the ICA

The ICA format, based on colloidal gold-based sandwich immunoassay, widely used for proteins or lower molecular weight analytes, appeared also to be efficient for *A. minutum*. After screening on several specific mAb raised against the surface of *A. minutum*, two antibodies were chosen for this assay: AMI11 as the capture antibody at the T line, and AMI6 as the revelation antibody (Fig.1). The biochemical nature of their antigens is as yet unknown and might prove to be a substantial task.
Dinoflagellates and more precisely the *Alexandrium* genius are rather large unicellular organisms with cell diameter varying from 10 to 15 µm. In addition to possible steric limits to diffusion in the porous material of the ICA, nitrocellulose is known to bind proteins strongly due to both hydrophobic and electrostatic interactions. Preliminary experiments showed from the intensity of the red line that algal cells migrate freely across the wide pore (15 µm) nitrocellulose membrane and much better than across pore size of 5 µm and 10 µm that gave a very low signal on the red line (data not shown). The structural integrity of *A. minutum* immobilized on the nitrocellulose membrane (15 µm) was checked by Scanning Electron Microscopy (SEM) analysis. The cell morphology appeared identical to that frequently reported (data not shown).
Thanks to the autofluorescence of *A. minutum* cells could be tracked under fluorescence microscopy. At an excitation wavelength of 460 nm-500 nm *A. minutum* exhibited a red fluorescence. The cells on the sample pad were initially visualized with red fluorescence signal and whole cell morphology (Fig.2A) with no fluorescence on the nitrocellulose membrane (Fig.2B). After waiting for the assay buffer to move into the absorbent wicking pad, cells with the same fluorescence and morphology were found on the nitrocellulose area (Fig.2B'). We also controlled that the quantity of cells deposited was decreased on the sample pad (Fig.2A'). This cannot be carried out with denaturized cells or lysated cells which have no significant fluorescence emission under the same conditions (data not shown). Then we checked the ICA assay by loading 50,000 whole cells (50 µL of a 10^6 cells/mL suspension) on the sample pad (Fig.2C), giving rise to a clear signal, without streaking or high background effects (Fig.2C'). The initial design was for whole cells but we could also show that it accommodates lyzed cells (Fig.2D and Fig.2D'). This robustness might be important in field applications where seawater samples could be filtered or treated later than sampling on site. To our knowledge, ICA are suitable for use under a large range of working conditions, providing known interferents of antigen-antibody interactions are absent or removed.

3.2 Sensitivity and stability

We prepared several dilutions from the 10^6 cells/mL stock suspensions in seawater to investigate the ICA performance in the range 50,000 to 500 cells per sample (Fig.3). The optical signal is clearly visible down to 25000 cells per sample, a faint band remaining visible below 2500 cells. Thus, the ICA is suitable with some bloom samples but would require a concentration step for most environmental samples. The alert level in France, established by REPHY French network created by IFREMER (Institut Français de Recherche pour l'Exploitation de la MER), is within the range 10^4-10^6cells/L for *A. minutum* depending on the coastal site. The stability of the assay was examined by testing the strip after 2 months of storage at room temperature. The performance of the ICA proved stable over that period (data not shown).
Recent improvements in labelling methods in ICA for enterotoxin detection have been described, e.g. the substitution of colloidal gold for fluorescent liposomes which allows a 15-fold enhancement of the

sensitivity (Khreich et al., 2008). Although these experiments were not performed in the present study, difficulties in applying this detection approach to fluorescent algal cells are likely.

3.3 Specificity

The AMI6 antibody used in this assay was selected for being very specific of *A. minutum* (Gas et al., 2009). We nevertheless confirmed that AMI11 as the capture antibody, and AMI6 as the revelation antibody did not give rise to any false positive signals in the presence of several algae cells, toxic or not, such as *Heterocapsa triquetra, Alexandrium tamarense, Karenia mikimotoi, Scripsiella trochoidea* and also bacterial cells (*E.coli*), added in the ICA at up to 10^6 cells per sample (data not shown). No detectable cross-reactivity occurred with any of these micro-organisms, some of which are commonly found in similar biotopes as *A. minutum*. It is noteworthy that immunochemical methods such as ICA might help to discriminate some algal strains that might be mistakenly included during a classical light microscope count.

4. Conclusion

A one step immunochromatographic assay (ICA) format to detect the toxic algae *A. minutum* was developed. To our knowledge, this is the first time in this format. The assay provides a qualitative signal which could be used to detect *A. minutum* in seawater samples. Visual results of the test were in good agreement with the results of the whole cell ELISA that was developed in our laboratory. In addition no cross-reaction with other algae strains were observed. The assay is rapid (15 min), convenient and easy to use. Its sensitivity at around 2500 cells is much lower than our previous ELISA technique (10 cells). Nevertheless, it is still suitable for the abundance generally observed in blooming areas. If necessary, this relative lack of sensitivity could easily be overcome with a preliminary concentration of the sample, either by centrifugation or filtration. Even harsh conditions could be used for this pre-treatment since the ICA was shown to be similarly effective with intact or lyzed cells.
This assay should prove to be advantageous in shellfish farming and many other applications where proliferation of toxic algae needs to be monitored. Moreover, this versatile technique could be adapted to many other toxic algae.

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FIGURE captions

Fig.1: Cross-section of immunochromatographic strip

The sample is loaded onto the sample pad, and the detector molecule eg AMI6 mAb conjugated with colloidal gold deposited on the conjugated pad are solubilized. Capillary action then draws the fluid mixture up the sample pad and into the nitrocellulose membrane. At the test line (T) a specific antibody mAb AMI11 immobilized as a thin strip in the nitrocellulose then captures the complex. On the control line (C), a goat anti rat mAb captures excess AMI6 and should always show a visible line, otherwise the test is invalid and must be repeated. Excess buffer and reagents not captured will then move into the absorbent wicking pad.
Thus the appearance of two lines indicates a positive result, while a valid negative test produces only the control line.

Fig.2: *Alexandrium minutum* whole cells and cell lysates in immunostrip

100µL of algal samples (10^6 cells/mL) were loaded onto the sample pad. The red autofluorescence of whole algal cells was checked under fluorescent microscopy using an exciting filter 460 nm-510nm (A) without any background on nitrocellulose (B). After flow migration up to the absorbent pad a decrease of cells number on the sample pad (A') was observed and algal cells were visualized on the nitrocellulose near the absorbent pad (B').
Whole *A.minutum* cells, the integrity of which was evaluted under light microscopy (C) and were used as a sample in the immunochromatographic assay (C').
The disrupted and sonicated algal cells (D) were visualized by optical microscopy and tested in immunostrip (D'). The visual color line was observed for each condition both in control area **[C]** and in test area **[T].**

Fig.3: Immunostrip sensitivity

The different algal concentrations were loaded of 50µL in samples area [s] and the visual color line was observed for each condition both in control area **[C]** and in test area **[T]**.

A: The sample was only seawater and was used as a negative control.
B: Estimation of 50 000 whole cells of *Alexandrium minutum*
C: Estimation of 25 000 whole cells of *Alexandrium minutum*
D: Estimation of 2 500 whole cells of *Alexandrium minutum*
E: Estimation of 500 whole cells of *Alexandrium minutum*

Fig. 1

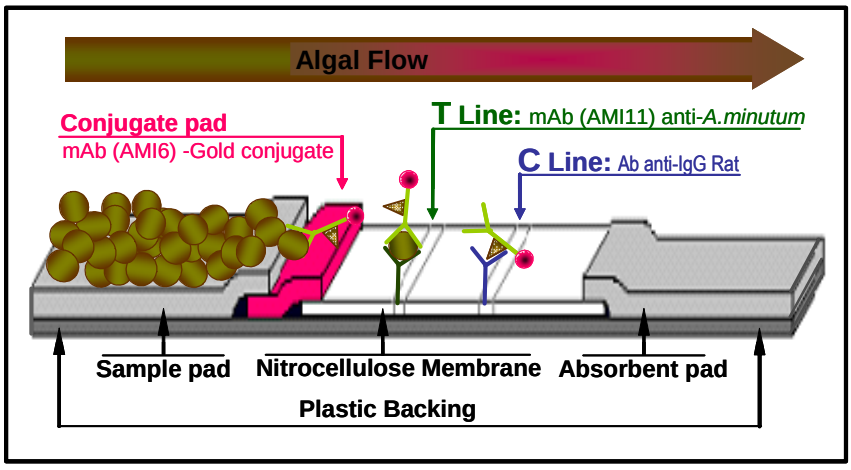


Fig. 2

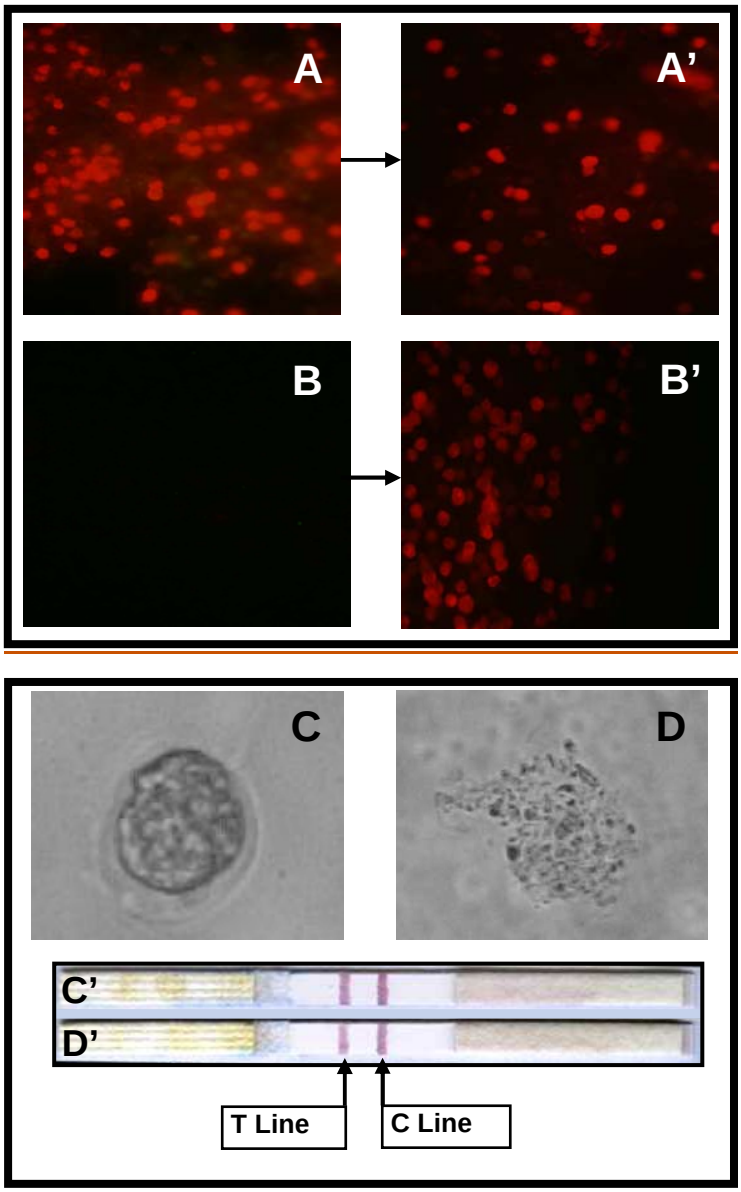
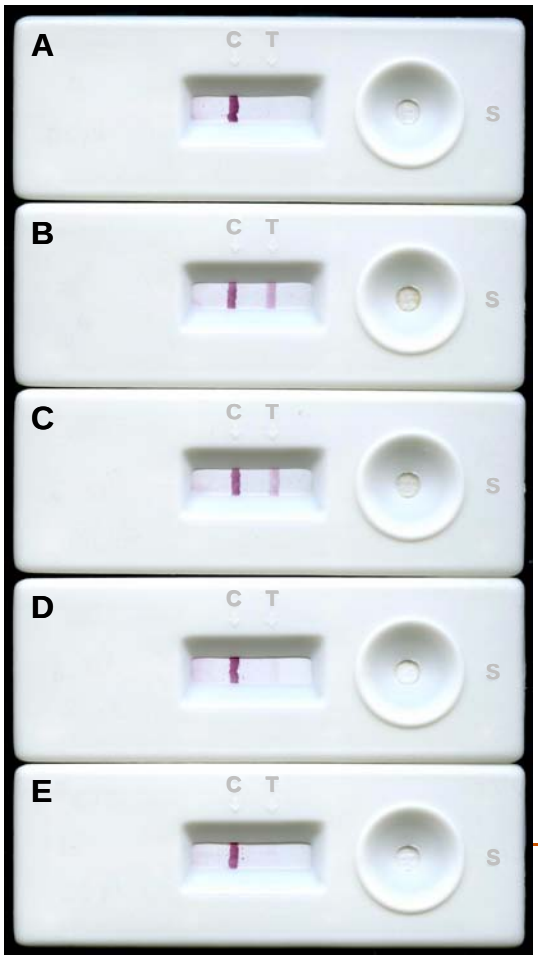


Fig. 3



Negative sample

50 000
A .minutum cells

25 000
A .minutum cells

2500
A .minutum cells

500
A .minutum cells