

IMMUNO-MAGNETIC SEPARATION FOLLOWED BY SOLID-PHASE CYTOMETRY FOR THE RAPID DETECTION AND ENUMERATION OF PATHOGENS IN SURFACE WATER

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INTRODUCTION: Contamination of surface waters with pathogens such *Naegleria fowleri*, *Cryptosporidium parvum*, *Legionella pneumophila* is a major public health concern worldwide. Detection and enumeration methods for these pathogens in water are based on culture enrichment to increase the concentration of viable organisms, followed by immunofluorescence assay, enzyme-immunoassay, polymerase-chain reaction, latex agglutination test, flow cytometry, or enzyme electrophoresis for identification and, to some extent, enumeration.

There are two major drawbacks for simple and rapid detection of pathogens in water. First, most of the current methods are not sensitive enough to detect small numbers of pathogens in their original environment. Second, technologies that could reach the required level of sensitivity are limited by the fact that large volumes of water (1 to 100 liters) must be processed for one single analysis. Membrane filtration often used is laborious, time-consuming and non-selective, leading to the presence in the filtrated samples of undesired contaminants such PCR inhibitors, auto-fluorescent events or cross-reactive unrelated organisms.

The ChemScanTM system (Chemunex, Ivry, France) is a recently developed solid-phase cytometer that allows the identification of rare events and their enumeration down to one pathogen or cell per analysis [1]. The ChemScanTM combines a fluorescent detection of membrane-filtered organisms with an automated counting system. To perform the detection of pathogens in large volumes with the ChemScanTM device, it is essential to use a simple and adapted sample preparation method in which contaminating elements are efficiently removed. To this end, immuno-magnetic separation (IMS) was utilized to recover pathogens directly from their water habitat, concentrating and selecting the organisms in a suitable volume for subsequent detection and enumeration by solid-phase cytometry.

However, procedures using IMS also present a major obstacle: detaching pathogens from magnetic beads prior to detection is generally not completely achieved. A significant number of captured organisms are likely to remain on the beads after the detaching step, leading to an erroneous enumeration. Using stronger elution conditions often has an impact on the integrity of the membrane of the pathogen, making the immuno-labelling uncertain.

Due to their low steric effects and their easy filtrability, the use of small sized magnetic particles (< 350 nm) makes possible the direct fluorescent detection of the whole complex bead-pathogen with the ChemScanTM system, so that a detaching step is not required.

In this report, we describe the development of an IMS technique for the rapid and efficient separation of *Naegleria fowleri*, a free-living amoeba found in various freshwater environments, known as the causative agent of a rare but almost always fatal meningo-encephalitis [2].

Several sizes and two natures of magnetic (ferrofluid and polystyrene) particles were compared for their compatibility with solid-phase cytometry analysis and their efficiency in amoeba recovery.

Both experimental (amoeba from culture) and natural samples were assayed.

Preliminary results on the detection and enumeration of *Cryptosporidium parvum* using the same technology are also discussed.

METHODS:

Samples: (*Naegleria fowleri*): Contaminated water samples were collected in the cooling system of nuclear power plants at different sites. Liquids were fixed with formaldehyde 2% and stored at +4°C. Experimental samples were obtained by adding amoeba harvested from cultures dishes in Ringer's solution.

Immunomagnetic separation of *Naegleria*

fowleri: Both indirect and direct immunoseparation were investigated. Magnetic particles from different origins (diameter 0.05 (ferrofluid), 0.19, 0.34, 1.05 and 2.8 μm) were sensitized with anti-*Naegleria fowleri* monoclonal antibody [3] or anti-*Cryptosporidium parvum* polyclonal antibody (Waterborne Inc., USA) for direct immunocapture. Indirect immunocapture was performed by using magnetic particles (0.34 μm) sensitized with anti-rabbit IgG polyclonal antibody.

Direct immunoseparation: 50 μl of each suspension of particles at 1% solid were added to 10 ml of contaminated water and incubated for 30 min. at room temperature. Particles were separated by using a Neodyn-Iron-Bore magnet of 1 tesla, washed twice with PBS, and filtered through a polycarbonate membrane (porosity 2 μm , diameter 25 mm).

Indirect immunoseparation: 10 μl of rabbit polyclonal antibody to *N.fowleri* (4.2 $\mu\text{g/ml}$) was added to 10 ml of contaminated water, and incubated for 30 min at room temperature. 50 μl of anti-rabbit IgG magnetic particles at 1% solid was added to the suspension and incubated for 15 min. After washing, the particles were filtrated through the 2 μm membrane. The immobilized *N.fowleri* were then revealed as described below.

Controls: Supernatants resulting from magnetic particle separations (containing non-captured organisms) were analyzed. Water samples not incubated with magnetic particles were also filtered and analyzed as positive controls.

Immunofluorescence staining: [4] Amoeba were revealed by incubating the membrane with biotinylated monoclonal antibody to *N.fowleri* (5D12), followed by incubation with streptavidin conjugated to R-phycoerythrin-Cy5.

Detection and counting: [4] All information regarding the ChemScanTM system can be obtained from the manufacturer. Briefly, the labelled membrane was transferred onto the sample holder of the ChemScanTM, and scanned by a water-cooled argon laser emitting at 488 nm. Emission was collected in the red channel (655-705 nm) for *Naegleria fowleri*, and in the green channel (500-530 nm) for *Cryptosporidium parvum*. After scanning the whole membrane, a set of discriminants was applied, allowing the differentiation between labelled amoeba and autofluorescent elements. Additionally, all positively selected events were manually validated by microscopic examination.

RESULTS:

Table 1. Comparison of direct and indirect immunocapture (0.34 μm particles).

	Direct	Indirect
Recovery (exp ^{al} sample)	100%	96%
Recovery (natural sample)	73%	1%

Table 2. Effect of particle size on the efficiency of the recovery of amoeba from experimental samples.

Particle size (μm)	0.05	0.19	0.34	1.05	2.8
Recovery	100%	99%	100%	100%	60%

Table 3. Effect of particle size on the efficiency of the recovery of amoeba from natural samples

Particle size (μm)	0.05	0.19	0.34	1.05	2.8
Recovery	89%	69%	67%	NI	NI

NI – Non Interpretable (no filtration possible)

Table 4. Effect of antibody coverage on the efficiency of the immunocapture (ferrofluid 50 nm).

Ratio Antibody/Particle	5	10	15
Recovery	50%	89%	71%

Table 5. Influence of particle binding on fluorescence intensity (ferrofluid 50 nm).

	Peak Intensity
Free amoeba	5.720 +/- 1487
Particle-bound amoeba	11.017 +/- 3811

DISCUSSION & CONCLUSIONS: Finalizing an immuno-magnetic separation method depends on numerous parameters such antibody coverage, coupling procedure, nature and size of the particle, type of sample, ratio of particle/pathogen, etc. As shown in this paper, most of the difficulty comes from the fact that, unlike experimental samples containing cultured organisms, natural samples are complex and often not suitable for immunoassay.

The first example is given by the comparison of direct and indirect immunocapture. Table 1 clearly demonstrates that, although the percentages of recovery are acceptable when using experimental samples, the indirect method is not suitable for the capture of amoeba in their natural environment (1% versus 96 %). This is partially due to the fact that the free monoclonal antibodies, when diluted in natural water, do not easily reach their target, due to unphysiological conditions. All other experiments were undertaken with the direct procedure.

As previously mentioned, most of the IMS reagents commercially available require a detachment step to separate the particles from the pathogen prior to identification. This often results in a significant loss of pathogens. In our example (Tables 2 and 3), the suspensions containing both particles and pathogens were filtered and labelled.

This requires small-sized particles. Indeed, during the filtration through a 2 µm membrane, the non reactive particles must pass through the membrane, while only particles bound on pathogens remain on the membrane. In this way, large volumes of sample can be processed.

One other condition that must be considered when doing IMS without a detachment step is that the binding of particles onto the membrane of the pathogen leaves sufficient antigenic sites open for subsequent immunofluorescent labelling. Table 5 surprisingly shows that when using 50 nm particles, the level of the fluorescent intensity is higher than that of free amoeba. One explanation that can be put forward is that the interaction of small size particles with cells may protect their integrity during the filtration step, thus giving a higher apparent immuno-reactivity.

Our study demonstrates that IMS coupled to ChemScan™ analysis is efficient in recovering and detecting amoeba as well as cryptosporidium from their water environment. The preliminary performance of this method appears to be sufficient for its future use in routine monitoring of surface waters.

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ACKNOWLEDGMENT: This work was supported by a grant from Electricité de France.