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Flow cytometry and RT-PCR for rotavirus detection in artificially seeded oyster meat

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Abstract

A flow cytometry (FC)-based method was developed for the detection of rotavirus in oyster meat using simian rotavirus SA11 as a model. To study virus recovery, oyster meat was injected with rotavirus and the oyster extract used to infect MA104 cell monolayers. Following varying periods of infection, the cells were recovered and reacted with the monoclonal antibody M60 which is specific for the rotavirus group A serotypes 1–4 outer capsid protein, VP7, followed by a second antibody (anti mouse IgG-FITC). A FACScan® FC was used to estimate the number of infected cells as well as the level of infection. To evaluate the sensitivity of the method, non-inoculated oysters were processed following the same extraction protocol and, at the end, they were seeded with the same amount of virus used for oyster inoculation. This seeded oyster extract was then used to infect MA104 cells and the number of infected cells determined using the same FC procedure. A semi-nested two-step PCR for detection of rotavirus nucleic acid was undertaken to compare the sensitivity of FC with RT-PCR. Using FC, as little as 0.02 flow cytometry units (fcu) (number of infected cells counted by FC) could be detected after 72 h of cell infection. This is a very similar limit of sensitivity to that obtained with RT-PCR. Both methods are approximately 100 times more sensitive than the plaque-forming units (pfu) assay.

Keywords: Flow cytometry; Oysters; Rotavirus; RT-PCR

1. Introduction

Rotaviruses are considered the major agents of severe infantile diarrhoea (Estes et al., 1983) and their presence has been described in drinking water (Deetz et al., 1984; Strappe, 1991), sea water (Goyal

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and Gerba, 1983) and shellfish (Lewis and Metcalf, 1988; Hafliger et al., 1997). Shellfish are readily contaminated with viruses present in water containing sewage because of the concentrating effect of filter feeding (Cromeans et al., 1997). Since viruses do not replicate in shellfish tissues, the vector potential of shellfish is considered to be due to the stability of viruses in their tissues. The risk of acquiring a shellfish-borne viral disease is substantial since they are often eaten raw, including the intestinal tract (Bouchriti and Goyal, 1993).

The polymerase chain reaction (PCR) has been used to explore various aspects of environmental microbiology by detecting nucleic acids of target organisms. After extensive sample preparation, the high specificity, sensitivity and consistency of the PCR has provided a powerful tool for virus detection in such samples (Bej and Mahbubani, 1994). PCR, in conjunction with reverse transcription (RT) can detect as little as 0.01 plaque forming units (pfu) per sample (Schwabb et al., 1995), which is equivalent to approximately 10 virus particles (Grinde et al., 1995). However, despite the sensitivity, contaminating substances in the environmental sample can severely limit the usefulness of this approach. For example, various proteins, carbohydrates, and other organic compounds may bind magnesium ions and nucleotides required by nucleic acid polymerases, and some solutes may be toxic to these polymerases (Schwabb et al., 1995; Wilson, 1997). Furthermore, positive PCR products can be amplified from the RNA of not only intact viable viruses, but also from non-infectious single-shelled particles or from liberated RNA of lysed viruses (Chen and Ramig, 1992; Limsawat and Ohgaki, 1997). This means that the quantitation of a PCR product cannot necessarily be equated to the number of infectious organisms present in the original sample (Vaneechoutte and Van Eldere, 1997). For public health authorities interested in environmental monitoring, the presence of non-infectious virus particles is of little public health significance (Smith and Gerba, 1982).

Flow cytometry (FC) is a well-established quantitative technology in medical diagnosis and the main advantage of this method in addition to quantitation, is the speed of analysis (10^2 to 10^3 cells can be analysed per second). Furthermore, cell sorting can physically purify defined populations for further analysis (Wallner et al., 1997). Vesey et al. (1994) have described FC methods for the detection of specific microorganisms in environmental samples. Recently, using clinical and water samples, Abad et al. (1997) described the detection of infectious human rotavirus using a Coulter Elite® FC. A similar study was undertaken using a FACSCan® FC (Barardi et al., 1998). Here we describe a very sensitive method for rotavirus detection in oysters using the FACSCan® FC method to determine infectious virus recovery from seeded oysters. We also compare the sensitivity of this method with RT-PCR.

2. Materials and methods

2.1. Virus and cells

Simian Rotavirus SA11 was propagated and assayed by FC in MA104 cells (donated by Dr G. Both, CSIRO, Sydney, Australia) as previously described (Barardi et al., 1998). The cells were cultivated in 12-well microplates (Corning, USA) with Eagle's minimal medium (MEM) supplemented with 1× non-essential amino acids, 15 mM Hepes buffer, pH 7.2, 2 mM glutamine and 10% (v/v) fetal calf serum. For rotavirus quantitation using FC, wells containing a confluent monolayer of MA104 cells ($0.8–1.7 \times 10^6$ cells/ml) were washed twice with phosphate-buffered saline (PBS) containing 150 mM NaCl, 1.5 mM KH_2PO_4, 20 mM Na_2HPO_4, 27 mM KCl, pH 7.2) and then each well was incubated with 300 μl of rotavirus SA11 harvested from infected culture supernatants. All assays were undertaken in triplicate. After 1 h of incubation at 37°C for virus adsorption, the inoculum was removed and the cells were supplied with serum-free MEM containing 5.0 μg/ml of trypsin. For virus quantitation, the maintenance medium was removed by suction between 2 and 10 h after infection. An aliquot of 0.1 ml of trypsin Versene (1:250, 20 μg/ml) was added to each well. After 10 min at 37°C, the detached cells were recovered, resuspended in 1.0 ml PBS and transferred to an Eppendorf tube. The cells were then counted in a haemocytometer chamber, and prepared for FC. The method described here for rotavirus detection in oysters has also been used for rotavirus

quantitation. In our previous paper (Barardi et al., 1998), we established the flow cytometry unit (fcu) as a new unit to quantitate rotavirus using flow cytometry. The sum of the number of infected cells counted by flow cytometry following inoculation with a defined dilution of virus plus the number of lysed cells, gives the number of fcu present in the sample. The method is very sensitive and can detect infected cells in very early stages of virus replication. We estimate that one fcu can corresponds to between 10 and 100 infectious viral particles.

2.2. Oyster contamination

Sydney Rock Oysters, *Saccostrea commercialis*, were obtained from a domestic market. The oyster shells were scrubbed with a stiff brush in running potable water and allowed to depurate in slow running tap water for 24 h. The surface of the shells was coated with Betadine® (Faultings) solution and left at room temperature in a Class II safety cabinet to air dry for 30 min. The shells were opened at the hinge with a sterile oyster knife. In the majority of cases, each oyster was seeded with 250 μl of rotavirus-infected tissue culture supernatant using a micropipette tip inserted at three points of the visceral area as previously described (Cromeans et al., 1997). In this case we used 1×10^6 flow cytometry units (fcu), to infect each oyster. After adsorption for 30 min at room temperature, the oyster extract referred as the 'pre-extraction' seeded oyster extract, was prepared.

2.3. Oyster extract preparation

Two techniques were compared for their effectiveness in infecting MA104 cells for the FC assay. *Method I* (Lewis and Metcalf (1988), with minor modifications): the flesh of two oysters (12–20 g) (untreated or 'pre-extraction' seeded with rotavirus) was transferred to a sterile 200 ml Schott® bottle (Crown Scientific) containing 100 ml of pre-chilled 10% (v/v) tryptose phosphate broth (TPB) (100% TPB contains, 20 g Tryptose, 2.0 g glucose, 5.0 g NaCl and 2.5 g Na_2HPO_4, pH 7.3) prepared in 0.05 M glycine (pH adjusted using 2 N NaOH). The tissues were homogenized with a shaft blender Ultra-turrax T-25 Ika® (Jenke Kunkel Labortechnik) at 24 000 rpm for 30 s. The resulting suspension was placed in a flask, shaken at 250 rpm at 22°C for 30 min and the contents centrifuged at $10\,000 \times g$ for 30 min at 4°C. The pellet was discarded and the pH of the supernatant adjusted to 7.5 using 2M HCl. Polyethylene glycol solution (PEG, 6000) (50%, w/v) prepared in 10% TPB was added to a final concentration of 8% (w/vol). The mixture was stirred for 2 h at 4°C and centrifuged at $10\,000 \times g$ for 20 min at 4°C. The pellet was resuspended in 5.0 ml of 0.15 M Na_2HPO_4, pH 9.0, sonicated for 30 s, shaken for 20 min as described above and recentrifuged at $10\,000 \times g$ for 30 min. The supernatant was transferred to a fresh tube and the pH readjusted to 7.4. Streptomycin, penicillin, amphotericin B and trypsin were added to final concentrations of 150 μg/ml (both penicillin and streptomycin), 50 μg/ml and 10 μg/ml, respectively. This preparation was designated oyster extract I. *Method II:* the flesh of two oysters (12–20 g) (untreated or 'pre-extraction' seeded with rotavirus) was blended in 100 ml of 0.2 M glycine, 0.15 M NaCl, pH 9.5 (pH adjusted using 2 N). Cat Floc® (polydimethyldiallylammonium chloride, Sigma (2.0 ml) was added to clarify the suspension by precipitating the oyster solids (Kostenbader and Cliver, 1972) and the solution shaken at 250 rpm for 5 min at 22°C. The preparation was sonicated twice for 60 s each and centrifuged at $10\,000 \times g$ for 15 min at 4°C. The aqueous phase was harvested, and 50% (w/v) PEG 6000 (Sigma) in 10 mM Tris, 100 mM NaCl and 3 mM $CaCl_2$, pH 8.0 (TNC buffer), was added to a final concentration of 8% PEG. The mixture was stirred for 2 h at 4°C and centrifuged for 15 min at $10\,000 \times g$ at 4°C. The PEG pellet was suspended in 30 ml TNC buffer pH 9.5 and the suspension sonicated for 30 s, shaken for 10 min at 250 rpm, and sonicated again for 30 s. The sample was then centrifuged for 30 min at $10\,000 \times g$ at 4°C and the pH of the supernatant adjusted to 7.5 with 2 M HCl. The sample was carefully overlayed onto 4.0 ml of 40% sucrose (w/v) in TNC buffer, pH 8.0, in a SW28 clear tube in a Beckman ultracentrifuge for 2 h at 4°C at 25 000 rpm in the SW 28 Beckman rotor. The final pellet was resuspended in 5.0 ml of TNC buffer, pH 8.0. Antibiotics and trypsin were added as for Method I and this solution was designated oyster extract II.

2.4. Cell infection

Oyster extracts I and II as well as virus control (4.5 ml of maintenance medium seeded with 500 μl of rotavirus, 2.0×10^6 fcu) were used for MA104 cell infection. To study the sensitivity of the method for both PCR and RT-PCR, unseeded oysters were extracted using Method I and then the oyster extract was seeded with an equivalent amount of virus as used for the 'pre-extraction' seeding. This preparation was referred to as 'post-extraction' seeded oyster extract. Unseeded oyster extracts were used as negative controls for cytotoxicity assays. MA104 monolayers were prepared in 12-well microplates (Corning®, USA) 48 h before the experiment and grown to a density of 1.0×10^6 cells/ml. Infection was carried out as follows: serial dilutions of oyster extracts and virus control in the range of 10^{-2} to 10^{-7} were prepared in maintenance medium. Aliquots of 500 μl were then adsorbed to MA104 cells previously washed with PBS. All assays were undertaken in triplicate. After 1 h incubation for virus adsorption, the inoculum was removed and the cells supplied with 1.5 ml serum-free MEM supplemented with 10 μg/ml of trypsin. It was found empirically that the optimum concentration of trypsin used in this assay (10 μg/ml) was greater than the level of 5.0 μg/ml determined when the method was developed for clean samples (Barardi et al., 1998).

2.5. Cell preparation for FC

At various times between 24 and 96 h of incubation in serum-free MEM supplemented with 10 μg/ml of trypsin at 37°C, the maintenance medium was removed by suction. Trypsin–Versene (1:250, 20 μg/ml) was added to each well (100 μl). After 10 min at 37°C, the detached cells were recovered, resuspended in 1.0 ml of PBS, transferred to an Eppendorf® tube and counted in a haemocytometer, to define how many cells/ml were used in the assay. At this stage 50 μl of cell suspension were stored at −70°C for RT-PCR. The cells were centrifuged at 4000 rpm in an Eppendorf® minicentrifuge for 3 min at room temperature and the pellet resuspended in 100 μl of PBS. After resuspension, 900 μl of chilled methanol was added. The cells were incubated for 5 min at room temperature on a rotator, centrifuged again and resuspended in 200 μl of PBS containing 1% (w/v) bovine serum albumin (Sigma) as blocking agent and 0.05% (v/v) Tween-20 to stop cell clumping and reduce non-specific binding of the Mab (blocking solution). The tubes were incubated on a rotator for 30 min at room temperature. After incubation, 200 μl of tissue culture supernatant containing Mab M60 were added (1:1 dilution in blocking solution) (Shaw et al., 1986) and incubated for 4–16 h at room temperature on a rotator. The cells were washed once in blocking solution and incubated at room temperature for 15 min with a goat anti-mouse IgG antibody conjugated with FITC (Silenus Laboratories, Hawthorn, Australia) (1:100 dilution) prepared in blocking solution and filtered (0.22 μm) prior to use. After centrifugation at 4000 rpm, for 3 min, the cells were resuspended in 400 μl of blocking solution and analysed using FC.

2.6. Flow cytometry

A Becton-Dickinson FACScan® flow cytometer was used to analyse all samples. The forward scatter (FSC) detector was set at E-1 and the side scatter (SSC), FL1 and FL3 detectors set at 220, 400 and 643 V, respectively. The threshold was set at an FSC value of 540. Logarithmic amplifiers were used for all detectors. A region (R1), that contained the cells, was defined on a dot plot of FSC (*x*-axis) and SSC (*y*-axis). Region R1 was then used to gate a second dot plot of FSC (*x*-axis) and FL1 (530 nm) (*y*-axis). Quadrants were defined such that 99.9% of uninfected control cells were located in the lower right quadrant. The horizontal line in the dot plots was defined as the cut-off between infected (above) and non-infected (below) cells and was obtained by the analysis of uninfected control oyster extracts (Fig. 1B). Only levels of infection above 5.0% were considered significant for rotavirus infection. A total of 5000 cells were analysed for each sample. The total number of infected cells was estimated by adding the number of lysed cells (subtracting the number counted from harvested cells after infection, from the number counted after incubation with uninfected oyster extract) together with the number of infected, non-lysed cells measured by FC.

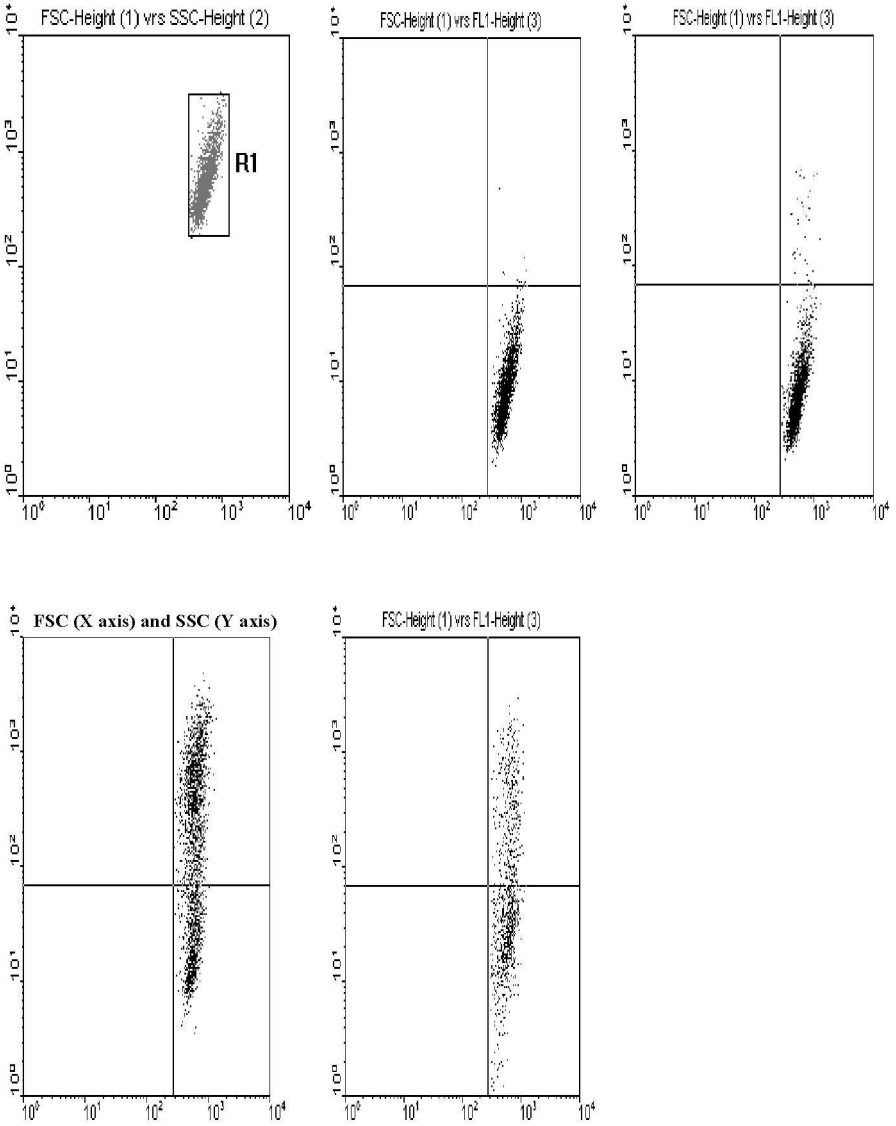

Fig. 1. Dot plot analysis of 5.0 ml of oyster extracts seeded with 2.0×10^6 fcu of rotavirus and diluted 10^{-3} for cell infection (0.5 ml used for each infection), corresponding to 2×10^2 fcu of virus input used for MA104 infection. (A) Using the unseeded oyster extract, the cell population was defined (R1) on a dot plot of FSC (*x*-axis) vs. SSC (*y*-axis) and this region was used to gate a second dot plot of FSC (*x*-axis) vs. FL1 (*y*-axis) (530 nm) (B). The horizontal line in these dot plots is defined as the cut off between infected (above) and non-infected (below) cells and was obtained by the analysis of uninfected control oyster extracts. Dot plots show cells infected with oyster extracts seeded with the above virus amount, 24, 48 and 72 h after infection (C–E).

2.7. RNA extraction

One-tenth (0.5 ml) aliquot of the 'pre- and post-extraction' seeded oyster extracts (equivalent to 2×10^5 fcu) was used for viral RNA isolation. An equal volume of the lipid solvent, Trichlorotrifluoroethane (Aldrich Cat. No. 27036-9, Australia) was added to remove lipids from the aqueous oyster extract and the suspension centrifuged at 6000 rpm in a minicentrifuge (Eppendorf®) for 5 min at room temperature. The aqueous phase was transferred to a fresh eppendorf tube and Tris–HCl, pH 7.5, EDTA, SDS and

proteinase K added at final concentration of 10 mM, 5 mM, 0.5% (w/v) and 400 μg/ml, respectively.

The samples were incubated at 37°C for 30 min. Cetyltrimethylylammonium bromide (CTAB; Sigma) and NaCl were added to give final concentrations of 1.3% (w/v) and 0.52 M, respectively, and the samples incubated at 56°C for 30 min. Subsequently, the samples were extracted twice with an equal volume of phenol–chloroform (1:1), and the aqueous phase was precipitated in 3 vol of ethanol. The resulting pellets were washed with chilled 70% (v/v) ethanol and suspended in 50 μl of RNAse-free water (Promega) and stored at −20°C for RT-PCR.

2.8. RT-PCR

The level of rotavirus detection by RT-PCR was first determined by using 2-fold serial dilutions of RNA ranging from 1:5 to 1:80 000 and corresponding, respectively, to 4×10^4 to 2.5 fcu. The oligonucleotide primers BEG9 (5′-GGCTTTAAAAGAGAGAATTTCCGTCTGG-3′) and END9 (5′-GGTCACATCATACAATTCTAATCTAAG-3′) (Gouvea et al., 1990), which produce full-length copies of gene 9 from any group A rotavirus strain, were used for the RT and first PCR. The primers ET3 (5′-CGTTTGAAGAAGTTGCAACAG-3′) and END9, were used for the semi-nested PCR (second amplification) (Gouvea et al., 1990). Five-μl aliquots of diluted RNA and 100 pmol of BEG9 and END9 primers were heated at 99°C for 5 min and chilled on ice for 1 min. The denatured RNA was added to the reaction mix consisting of 20 mM Tris–HCl, pH 8.4, 50 mM KCl, 0.4 mM each dATP, dCTP, dTTP and dGTP, 1.5 mM $MgCl_2$, 10 mM DTT and 50 U of Superscript II™ RT. The tubes were incubated at 42°C for 50 min, 70°C for 15 min and cooled to room temperature. A total of 1 U of RNAse H was added and the tubes incubated at 37°C for 20 min. After this step, 2.5 U of Taq polymerase (Promega) were added and the tubes overlayed with mineral oil and placed in a thermocycler (Omnigene) for 25 cycles of PCR (94°C for 1 min, 55°C for 2 min and 72°C for 3min) and a final 10-min incubation at 72°C. For the semi-nested PCR, 2.0 μl of the first PCR product was used for a reaction mix of 50 μl containing 20 mM Tris–HCl, pH 8.4, 50 mM KCl, 100 pmol of ET3 and END9 primers, 1.5 mM $MgCl_2$, 0.2 mM dNTP and 2.5 U of Taq DNA polymerase. The same PCR program was used.

To perform RT-PCR without RNA extraction, a PCR program described by Grinde et al. (1995), was followed with modifications. An aliquot (50 μl) of cells infected with ‘post-extraction’ seeded oyster extract 1, was freeze-thawed three times, centrifuged at $4000 \times g$ for 10 min and 7.0 μl of the supernatant used directly for RT-PCR. The disrupted cells were heated for 8 min at 99°C in the presence of 4 mM DTT, 2 mM EDTA and 100 pmol of BEG9 and END9 primers in 12.5 μl final volume. The tubes were immediately transferred to ice and mixed with 12.5 μl solution to make the final concentrations during RT: 20 mM Tris–HCl, pH 8.4, 50 mM KCl, 3 mM $MgCl_2$, 2 mM DTT, 1 mM EDTA, 0.5 mM dNTP and 50 U of Superscript II™ RT. The same conditions already described were followed for RT. For the first PCR, 25 μl of a solution containing 20 mM Tris–HCl, pH 8.4, 50 mM KCl, 100 pmol of BEG9 and END9 primers and 0.5 U of Taq polymerase were added to the cDNA. The tubes were transferred directly to 94°C and the PCR run for 40 cycles (94°C for 50 s, 55°C for 60 s and 70°C for 50 s) with a final incubation at 70°C for 10 min. This PCR program was especially used for rotavirus infected fluid without the need of RNA isolation. For the semi-nested serotype specific (G3) PCR the same conditions described above were used.

3. Results

3.1. Evaluation of rotavirus recovery from oyster extracts using FC

It was evident that undiluted oyster extracts obtained by either protocol (and with no virus present) were toxic to the MA104 cells, killing them within a few hours of contact, whereas a 1:64 dilution of the oyster extract did not cause any visible signs of toxicity in MA104 cells (data not shown). The percentage of infected cells detected by FC following inoculation of MA104 cells with virus directly in maintenance medium was considered as a reference for 100% recovery of added virus.

‘Post-extraction’ seeded oyster extract was also used as a control for monitoring recovery versus cytotoxicity of the oyster extract. Two methods of

oyster extraction were compared for virus recovery. Method I was both simple and quick and based on using TPB as virus eluant and PEG for virus precipitation. On the other hand, Method II was more complicated and based on the use of alkaline pH and high salinity for virus elution (glycine/NaCl), and Cat-Floc®, which promotes precipitation of the solids enabling them to be removed easily by centrifugation thus reducing cytotoxicity. This was followed by the use of PEG precipitation and a sucrose cushion to improve the purity of the oyster extract. After 48 h of cell infection, little difference in recovery was observed between these two methods (Table 1). For both experiments in which input virus was 2×10^6 fcu per 12–20 g of oyster meat, the average recovery of triplicate experiments was 86%.

3.2. Sensitivity of virus detection using flow cytometry

To determine the sensitivity of virus detection by FC, oyster extracts prepared according to Method I were serially diluted in maintenance medium and used to infect MA104 cells as described in Section 2. The percentage of infected non-lysed cells was measured by FC. When the virus concentration was too high, the percentage of infected cells measured by FC decreased in longer periods of incubation because of the cell lysis caused by the virus infection. The total number of infected cells was estimated by adding the number of lysed cells (subtracting the number counted in time zero of infection by the number counted after each different period of infection) together with the number of infected, non-lysed cells measured by FC. On the other hand, following inoculation with a low concentration of virus, prolonged incubation resulted in an increase in the percentage of infected cells measured by FC. After a period of 72 h of cell infection, virus was consistently detected in the oyster extracts at a dilution of 10^{-7} which is equivalent to 0.02 fcu (as 0.5 ml of each dilution of oyster extract was used to infect the cells) (Table 2). Fig. 1 shows the dot plot patterns obtained with the FACSCan® of cells inoculated either with unseeded oyster extract (Fig. 1A,B) or with 2×10^2 fcu in oyster extract (corresponding to 10^{-3} dilution) and incubated for 24, 48 or 72 h (Fig. 1C, D and E, respectively).

Table 1
Recovery of rotavirus from oyster extracts 48 h post-infection using flow cytometry[a]

Sample	Number of cells/ml $\times 10^5$	Number of lysed cells/ml $\times 10^{5b}$	% Infected cells by FC	Number of infected cells by FC $\times 10^{4c}$	Lysed + infected cells $\times 10^5$	% Recovery
Negative control	9.7±0.7	0	NC	NC	NC	NC
Oyster extract (10^{-2})[d]	13±4.6	0	NC	NC	NC	NC
Virus control (10^{-2})[e]	0.88±0.04	12.1	29.4±2.7	2.6±0.2	12.4	100
Oyster extract seeded (10^{-2})[f]	0.54±0.03	12.5	46.6±5.2	2.5±0.3	12.8	103.2
Method I (10^{-2})[g]	4.2±0.3	8.8	23.1±1.0	9.7±0.04	9.8	79.0
Method II (10^{-2})[h]	2.2±0.4	10.8	23.2±1.8	5.1±0.4	11.3	91.1

[a] All the results are average of three experiments. NC, not considered.

[b] Number of lysed cells was calculated by subtracting the number of cells/ml found in uninfected oyster extract from the number of cells counted after each experiment, both counted by haemocytometer.

[c] Number of infected cells was estimated from the percentage of infected cells by FC.

[d] Unseeded oyster extract prepared according to Method I and diluted 10^{-2} to inoculate in the cells.

[e] Maintenance medium seeded with same volume of virus-infected fluid used for oyster infection (0.5 ml virus to 5.0 ml final volume) and diluted 10^{-2} before cell inoculation. In this case the reference of 100% recovery was adopted. Input virus was 2.0×10^3 fcu.

[f] 'Post extraction' seeded oyster extract prepared according to method 1, seeded with 2×10^6 fcu of rotavirus and diluted 10^{-2} before cell inoculation. Input virus was 2.0×10^3 fcu.

[g] 'Pre-extraction' seeded oyster extract purified according to Method I and diluted 10^{-2} before cell inoculation. Input virus was 2.0×10^3 fcu.

[h] 'Pre-extraction' seeded oyster extract purified according to Method II and diluted 10^{-2} before cell inoculation. Input virus was 2.0×10^3 fcu.

Table 2
Sensitivity of FC and RT-PCR for rotavirus detection after different times of MA104 infection using serially diluted oyster extracts seeded with rotavirus[a]

Dilution of oyster extract after virus seeding	Virus input (fcu)	% Infected cells by FC after 24 h	Result of RT-PCR after 24 h	% Infected cells by FC after 48 h	Result of RT-PCR after 48 h	% Infected cells by FC after 72 h	Result of RT-PCR after 72 h	% Infected cells by FC after 96 h
10^{-2}	2×10^{3}	5.9±0.7	+	70.1±2.5	NP	NC	NP	NC
10^{-3}	2×10^{2}	1.1±0.2	+/−	52.2±3.5	++	30.0±4.3	NP	NC
10^{-4}	2×101	0.1±0.1	−	15.1±8.0	+	17.7±4.0	NP	NC
10^{-5}	2×100	ND	ND	1.5±0.3	+	54.6±1.6	++	36.0±2.2
10^{-6}	0.2	ND	ND	0.6±0.1	+/−	31.0±1.1	+	24.9±0.8
10^{-7}	0.02	ND	ND	0.4±0.1	−	8.0±0.2	+	14.3±1.4
10^{-2}	0	0.1[b]	ND	0.1[b]	ND	0.1[b]	−	0.1[b]

[a] All numbers are average of three experiments. ND, not determined because of the low virus concentration at this period of incubation; NC, all the cell were lysed after this period of incubation with the mentioned virus input indicating high level of virus infection; NP, RT-PCR not performed; +/−, on RT-PCR means a very faint band visualised on PAGE; +, on RT-PCR a visible band on PAGE; ++, on RT-PCR a very strong band visualised on PAGE.

[b] 0.1% corresponds to the background of fluorescence measured by the FC.

3.3. Sensitivity of virus detection using RT-PCR

The sensitivity of the PCR for virus detection was first evaluated using a total RNA extract from the seeded oyster extract. The acidic polysaccharides and glycogen present in oyster extracts can be significant inhibitors of the PCR genome amplification. To reduce the effect of inhibitors, samples were processed by precipitation with cetyltrimethylammonium bromide (CTAB) and ethanol. Despite these precautions, PCR inhibitors were still present in the oyster extract since serial 2-fold dilution of the RNA in RNAse-free water to a dilution of 1:80 000 improved the genome amplification (Fig. 2A). The second PCR product (semi-nested) could be detected at a dilution of the RNA extract of 1:80 000 (equivalent to 2.5 fcu) (Fig. 2A).

To avoid interference with inhibitors and to compare the results of sensitivity obtained with FC, infected cells were heated to release the virus genome and this was used directly for RT-PCR (without the need for RNA purification). When the presence of virus infection was evident by FC, the RT-PCR was performed only in some of the samples (as positive controls). In some cases, the use of semi-nested PCR products gave sensitive results more quickly than FC for detecting virus-infected cells. For example, 0.2 fcu could be detected by semi-nested PCR but not by FC 48 h after infection (Fig. 2B; Table 2). However, in both cases, 0.02 fcu of virus input could be detected 72 h after cell infection (Fig. 2B; Table 2).

4. Discussion

Our results confirmed that FC is a very powerful technique for detecting microorganisms in environmental samples (Vesey et al., 1994). A measure of the significance of the FC method is its applicability not only to different kinds of clinical and environmental samples (Scillian et al., 1989; Hoffman et al., 1997; Imbert-Marcille et al., 1997), but also to viruses of demonstrated pathogenic potential that are transmitted via water or shellfish routes (Abad et al., 1997). This paper describes the establishment of a rapid and reliable procedure for the detection of viable rotavirus in artificially infected oysters.

The effectiveness of the FC method was determined by monitoring virus recovery following oyster extraction and the sensitivity of the method was studied by comparing the number of viruses detected by FC with that using the RT-PCR method. The brownish Betadine solution which was used for shell disinfection before shucking was a very good indicator of external disinfection but did not interfere with the oyster meat inside the shell.

Two procedures were compared for extracting virus from oyster tissue. There was no significant difference in the recovery of virus from oysters between the two methods. PEG 6000 proved effective as a virus concentrator (Lewis and Metcalf, 1988) and showed little toxicity. Hence, the simpler of the methods, Method I, which uses a TPB-based elution protocol for extracting virus from oyster, was adopted to study the sensitivity of the FC method. As

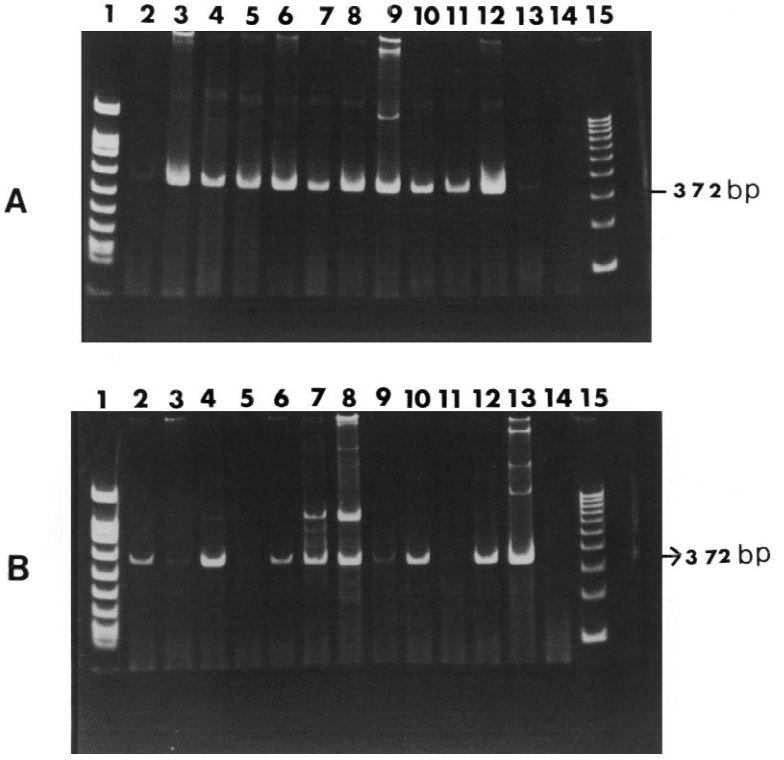

Fig. 2. (A) Detection of rotavirus in oysters extracts obtained from two oysters seeded with 2×10^6 fcu by use of RT-PCR followed by semi-nested PCR. Lanes 1 and 15, molecular weight markers; lanes 2–13, serial 2-fold dilution of RNA extract, 1:5, 1:20, 1:80, 1:320, 1:640, 1:1280, 1:2500, 1:5000, 1:10 000, 1:20 000, 1:40 000 and 1:80 000, equivalent to 5×10^4 fcu to 2.5 fcu; lane 14, negative control oyster extract. (B) Detection of rotavirus in MA104 cells infected with oyster extracts seeded with rotavirus by use of RT-PCR followed by semi-nested PCR. Lanes 1 and 15, molecular weight markers; lane 2, 10^{-2} dilution, 24 h p.i.; lanes 3 and 4, 10^{-3} dilution, 24 and 48 h p.i., respectively; lanes 5 and 6, 10^{-4} dilution, 24 and 48 h p.i., respectively; lanes 7 and 8, 10^{-5} dilution, 48 and 72 h p.i., respectively; lanes 9 and 10, 10^{-6} dilution, 48 and 72 h p.i., respectively; lanes 11 and 12, 10^{-7} dilution, 48 and 72 h p.i., respectively; lane 13, positive infected fluid, 72 h p.i.; lane 14, 10^{-2} dilution of uninfected oyster extract. Semi-nested PCR products were detected by using an ethidium bromide-stained 10% polyacrylamide gel. The equivalent virus input for each dilution can be found in Table 2.

the number of virus particles in the oyster extract decreases, the length of time that the MA104 cells are incubated following exposure to the infected oyster extract becomes more critical to ensure that infected cells will be detected by FC and RT-PCR. For the adapted SA11 rotavirus strain, we found that 72 h of infection was required to detect the lowest level of virus used for infection (0.02 fcu) in both methods. For wild-type rotavirus we expect that a longer period of incubation may be required in order to detect virus by FC and RT-PCR. This increased time is due to the lower replication time for environmental virus within the MA104 cells and the reduced likelihood for subsequent infection of neighboring cells.

Comparing the results obtained by FC, and RT-PCR followed by the semi-nested PCR detection, we confirmed that when RNA is extracted from the oyster extracts, inhibitors decrease the sensitivity of virus detection by RT-PCR. When the RT-semi-nested PCR was performed on infected cells following incubation with the oyster extract, the problem of inhibition was eliminated. There was no need to isolate RNA and the level of sensitivity was similar to that obtained by FC. In addition, in contrast to a PCR on RNA from the oyster extract, this approach, like FC, only detects viable viral genomes because the infected cells were used for the RT-PCR. The semi-nested PCR allowed the rotavirus strain to be genotyped as the internal primer is serotype-specific. As the FC described here uses monoclonal antibody M60 which is specific to group A rotaviruses, in this

case only the group A (serotypes 1–4) could be detected (Shaw et al., 1986).

This paper uses two powerful techniques for detecting low levels of viable rotavirus particles in oysters, both 100 times more sensitive than the pfu assay. We anticipate that, the sensitivity of the FC method for viable virus detection in environmental samples will be further improved by sorting single infected cells in the very early stages of virus replication, thus minimizing the need for virus adaption.

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