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International Journal of Food Microbiology 61 (2000) 177–185

INTERNATIONAL JOURNAL OF
Food Microbiology

www.elsevier.nl/locate/ijfoodmicro

Rotavirus survival and stability in foods as determined by an optimised plaque assay procedure

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Received 6 March 2000; received in revised form 31 May 2000; accepted 5 June 2000

Abstract

Tissue culture adapted rotavirus strains were propagated in MA104 and CaCo2 cells using standard cell culture procedures. The progress of infection was monitored by examining for a cytopathic effect, and for the presence of viral RNA in the tissue culture supernatant as determined by a guanidinium-based method. Subsequently, an effective plaque assay for rotavirus was developed using MA104 cells by optimising the adsorption time (2 h) and the levels of fetal calf serum (2.5%) in the overlay medium. Tragacanth gum was used in the overlay medium to immobilise the virus, and plaques were subsequently stained with 1% crystal violet. Using this optimised plaque assay, the survival of rotavirus following exposure to heat and UV irradiation was evaluated by enumerating the clear plaques. It was shown that 60°C for 10 min was sufficient to reduce the viral titer by at least 7 logs, and 50 mJ of UV irradiation was sufficient to reduce the initial viral titer by >2.5 logs. This optimised plaque assay was also used to determine the survival and stability of rotavirus from a range of experimentally contaminated foods including fruit juice, formula milk and lettuce.

Keywords: Rotavirus; Plaque assay; Recovery; Stability; Food

1. Introduction

Rotavirus is the single most reported cause of paediatric gastroenteritis worldwide and accounts for up to 873,000 deaths annually (Cimmons, 1998). Its importance as an enteric pathogen has been confirmed by recent rotavirus vaccine developments in the United States. Early detection and quantitation of the virus from clinical, food and environmental samples is important and can be performed using previously reported molecular methods such as enzyme immunoassays (Beards et al., 1984), hybridisation strategies (Flores et al., 1983; Fernandez et al., 1992) and the polymerase chain reaction (Xu et al., 1990; Hernandez et al., 1997). Furthermore, the virus

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0168-1605/00/$ – see front matter
PII: S0168-1605(00)00378-0

can be detected in samples by analysing for the presence of the rotavirus genome which consists of 11 segments of double-stranded RNA (electropherotyping) (Herring et al., 1982). However, although these methods exhibit excellent sensitivity and specificity, they are unable to discriminate between infectious and non-infectious viruses, a factor which limits their applicability.

Alternatively, rotavirus can be detected and infectivity can be determined following growth in cell culture (Wyatt et al., 1980), and many detection protocols have been developed which are based on rotavirus propagation (Ward et al., 1984; Hughes, 1993; Tietzova and Petrovicova, 1994). The African Green Monkey Kidney cell line MA104 has been shown to be the most appropriate cell line for supporting rotavirus growth (Ward et al., 1984), although the virus has also been grown in CaCo2, CV-1 and LLC-MK2 cell lines (Smith et al., 1979; Pinto et al., 1995). More recently, a combination of cell culture and molecular detection protocols have been employed to facilitate the sensitive detection of human rotavirus from a variety of sources (Pinto et al., 1994, 1995; Cumino et al., 1998), and the presence of infectious human rotavirus can also be determined using cell culture techniques in conjunction with immunofluoresence staining (Birch et al., 1983) or flow cytometry (Baradi et al., 1998).

The conditions for propagating rotavirus can be modified by titrating and immobilising infected virus with an overlay medium which localises viral infection in the form of discrete countable plaques. This 'plaque assay' is an adaptable and versatile technique which can be used to detect and enumerate infectious viruses. The composition of the overlay medium is critical, and the requirement for trypsin which is important for the reproducible formation of rotavirus plaques has previously been reported (Smith et al., 1979). A variety of immobilising agents in the overlay medium have been employed in the past for viral plaque assays, including agar (Urasawa et al., 1982), methylcellulose (Burke and Mulcahy, 1980), Sephadex G-75 (Aha and Sabara, 1990) and tragacanth gum (Jochim and Jones, 1976; Dobos, 1976; Manning and Collins, 1979). The main advantage of using this last agent is that it can be applied at room temperature and consequently does not result in possible heat-induced damage of cells commonly associated with agar (Dobos, 1976). Furthermore, Imammura et al. (1991) have shown that fetal calf serum (FCS) is an important component of the overlay medium as it plays a significant role in stabilising the monolayer.

By means of cell culture and plaque assays, rotavirus has been shown to remain viable on inanimate surfaces such as glass, plastic and stainless steel for more than 10 days at ambient temperature (Ansari et al., 1991), and has also been recovered from toilet handles, televisions, toys, charts and patients' hands (Akhter et al., 1995). Many waterborne outbreaks of rotavirus have been documented (reviewed by Ansari et al., 1991), and the virus has also been associated with food in the past (le Guyader et al., 1994; Cliver, 1996; Hernandez et al., 1997). Furthermore, using plaque assays, the stability of rotavirus has been examined (Estes et al., 1979), and the effects of temperature, relative humidity and ultra-violet light on rotavirus survival has previously been evaluated (Moe and Shirley, 1982; Chang et al., 1985).

The first reported rotavirus plaque assay was recorded by Smith et al. (1979) for simian rotavirus and since then substantial research has focused on the development and applications of this assay. In this report, we describe an efficient cell culturing model for rotavirus and describe a versatile plaque assay for enumerating infectious virus. Furthermore, we illustrate how this assay can be used to recover rotavirus from foods and evaluate viral stability following laboratory induced stress.

2. Materials and methods

2.1. Cell lines, viruses and infections

The tissue culture adapted rotaviruses Wa and DS1 used in this study were cultured in MA104 and CaCo2 cell lines. All cell lines originated from the European Centre for Animal Cell Culture (ECACC), and were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL, Paisley PA49RF, UK) which also contained 5–10% FCS, and 1× non-essential amino acids (Gibco). All cell lines were propagated in 25 or 100 cm^2 tissue culture flasks and six-well microtiter dishes (Costar, Cambridge, MA, USA) at 37°C in a humidified atmosphere containing 5% CO_2. When the cells were

confluent, the medium was removed and cells were washed with 1× PBS, and stripped from the growth surface by the addition of 0.25% trypsin in PBS (Gibco) at 37°C for 5–10 min. Subsequently, the cells were diluted with serum free DMEM and added to fresh flasks with a split ratio of 1:5. During infection, virus samples (generally 500 μl) were preactivated with 10 μg/ml of trypsin (Sigma) for 30 min at 37°C, serially diluted 10-fold in DMEM, and inoculated onto cells containing fresh DMEM and 5 μg/ml trypsin. The cells were incubated at 37°C with gentle agitation and monitored each day for the appearance of a cytopathic effect (CPE). Infectious virus was 'freeze–thawed' three times, harvested by centrifugation, filtered through 0.45 μm filters (Sartorius) dispensed and stored at −70°C.

2.2. Confirming infection

A cytopathic effect is indicative of cellular infection but does not confirm the presence of rotavirus. To establish the presence of rotavirus and monitor the progress of infection in CaCo2 cells, 10% of the tissue culture supernatant was removed each day post-infection and analysed for the presence of rotavirus RNA. Each sample was mixed with an equal volume of Ultraspec RNA isolation reagent (Biotecx Laboratories, Houston, TX, USA) and 5% chloroform (Sigma, St. Louis, MO, USA), vortexed for 30 s and left on ice for 15 min with intermittent mixing. The samples were centrifuged for 15 min at 10,000 *g*, the supernatant was decanted and mixed with an equal volume of ice cold isopropanol. The tube containing the RNA was gently inverted and left to precipitate at −20°C for at least 15 min. Subsequently, the samples were centrifuged for 15 min at 10,000 *g*, washed with 70% ethanol, dried in a sterile laminar flow hood and resuspended in 50 μl of DEPC-treated water. RNA was stored at −20°C until analysed on RNAse free 2% agarose gels as previously described (Sambrook et al., 1989).

2.3. Plaque assay

Tissue culture adapted viruses were diluted, preactivated and introduced to MA104 cells as described above. Following adsorption of the virus for 120 min at 37°C, the supernatant was removed and replaced with an overlay medium which consisted of an equal volume of 2× MEM, 1.5% tragacanth gum (Sigma), 2.5% FCS and 5 μg/ml of trypsin. The flasks were left for 2–4 days and examined for CPE. The overlay medium was removed and cells were stained with 1% crystal violet (in 99% ethanol) for 1 min. Subsequently, the stain was decanted, the cells were washed three times with sterile distilled water and the plaques were enumerated.

2.4. Optimisation of viral adsorption time and FCS concentration

Apart from the relative infectivity of the virus, the number and quality of plaques generated during this assay is directly related to the adsorption time and the composition of the overlay medium (Adsorption time refers to the length of time that the virus is allowed to adsorb to the cells before the supernatant is removed and the cells are overlayed.) To optimise this adsorption time, eight flasks of MA104 cells were grown to confluency and infected with 500 μl of a pre-activated 10^{-4} dilution of Wa virus simultaneously in triplicate. The virus in each flask was left to adsorb over a range of times (20–210 min), following which the infected cells were overlayed as before.

The importance of FCS in the overlay medium has previously been reported (Imammura et al., 1991), and the optimal levels of FCS required for this method were determined from conventional plaque assays which were set up with varying FCS concentrations (0–10%) in the overlay medium. Subsequently, the resultant number of plaques for each concentration was enumerated and compared.

2.5. Thermal stability of rotavirus

Four 500 μl samples of the Wa rotavirus strain were placed into sterile thin walled micro-centrifuge tubes. Three of these tubes were heated rapidly in a Hybaid Omnigene thermal cycler to 50°C and the time was noted. At 10 min intervals, a tube was removed, the virus was pre-activated and then serially diluted 10-fold in duplicate. Each dilution (including those from the fourth unheated tube) was plaqued as described above (adsorption time 120 min, 2.5% FCS) and thus the survival of rotavirus at 50°C was determined. Similar experiments were carried out at 55, 60 and 63°C. The temperature of the tubes was

regulated to within 0.2°C during each experiment by a thermocouple in a control tube.

2.6. Ultra-violet (UV) inactivation

Samples (500 μl) of Wa rotavirus were thawed and placed in duplicate into a well of a sterile six-well microtiter dish. Both samples were exposed to 10 mJ of UV light in a UV stratalinker (Stratagene), and the effect of UV light on the survival of rotavirus was determined by diluting and plaquing each pre-activated sample as described above. The procedure was repeated for 20, 30, 40 and 50 mJ of UV energy (260 nm).

2.7. Survival and recovery of rotavirus from fruit juice

A commercial fruit punch was filter sterilised and the pH was recorded. Ten-fold dilutions of DS1 virus (in 1 ml) were made in the filtered juice and left to stand at 4°C for 3 days. Meanwhile, similar amounts of the virus were also diluted in DMEM and left at 4°C as a control. After 3 days the virus containing juice and the control virus were plaqued to establish the survival of rotavirus in a cold low pH environment.

2.8. Survival and recovery of rotavirus in infant formula milk

A 500 μl sample of Wa rotavirus was serially diluted in DEPC water and mixed with an equal volume of 2× infant formula for 1 h. Each dilution was filter-sterilised and plaqued as described above.

2.9. Survival and recovery of rotavirus from lettuce

Lettuce leaves were cut into squares ca. 3 cm × 3 cm using a sterile scalpel and placed into the wells of a microtiter plate (six-well). A rotavirus sample was thawed (DS1), pre-activated and diluted serially 10-fold in DMEM. One hundred microlitres of each dilution was added to the strips of lettuce in duplicate and left to dry in a laminar flow hood for 4 h. Each strip was then washed in 5 ml of DMEM, and this was diluted and plaqued.

3. Results

3.1. Determining viral infection

The success of an infection was initially determined by the appearance of a cytopathic effect (CPE) in the monolayer of infected cells when compared to controls (Fig. 1a). Localised CPE usually appeared at the edges of the culture flask initially and subsequently developed into the middle of the monolayer over time. The rate of CPE development was related to the titer and the infectivity of the virus. It was noted that CPE appeared in MA104 infected cells more rapidly (generally overnight) when compared with CaCo2 cells (at least 1–3 days). Rotaviral infection was established and monitored over time by analysing tissue culture supernatant from infected monolayers for the presence of rotavirus RNA (Fig. 1b)

3.2. Plaque assay optimisation

The length of the adsorption time for rotavirus in MA104 cells was investigated and optimised by counting the number of plaques from each subsequent assay, the results of which are presented in Fig. 2. Based on these data all viruses were adsorbed for 2 h. The inclusion of FCS in the overlay medium has been shown to be critical for successful rotaviral infection and its concentration was optimised. The number and quality of the plaques generated with each FCS concentration were evaluated and indicated that the optimal concentration is 2.5% which was used in all subsequent plaque assays. An example of an infected flask following a plaque assay as described in the Materials and methods section is shown visually in Fig. 3a and microscopically in Fig. 3b. Tragacanth gum was used in the overlay medium at a final concentration of 0.75% which was sufficient to localise the viral infection and generate discrete plaques.

3.3. Thermal and UV stability of rotavirus

The applications of this optimised plaque assay were considered by investigating the effects of heat and ultra-violet light on the survival of rotavirus. An experiment was set up to simulate an LTLT pasteurisation cycle (63°C × 30 min). The initial titer of

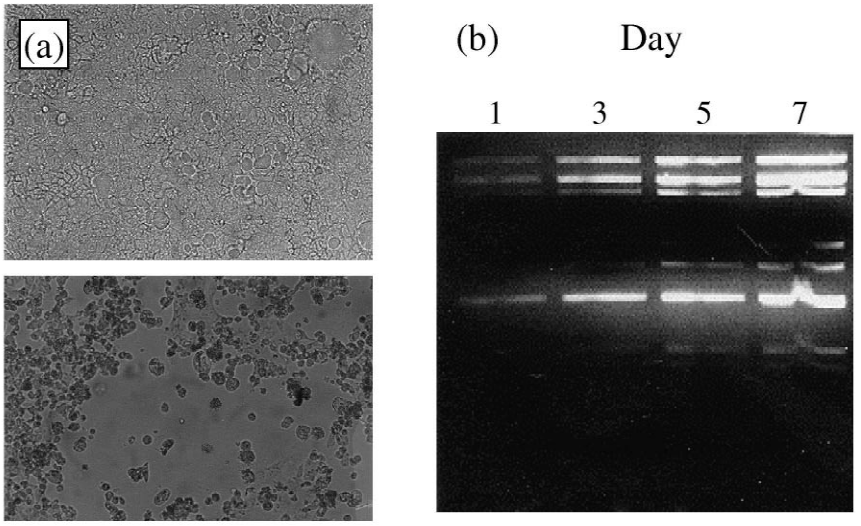

Fig. 1. (a) Comparison of an uninfected CaCO2 monolayer (top) with an infected monolayer (bottom) following a rotavirus infection (4 days). (b) Photograph showing an increase in rotaviral RNA from the infected supernatant of MA104 cells over a period of 7 days. Densitometry analysis illustrates over a 12-fold increase in viral numbers from day 1 to day 7.

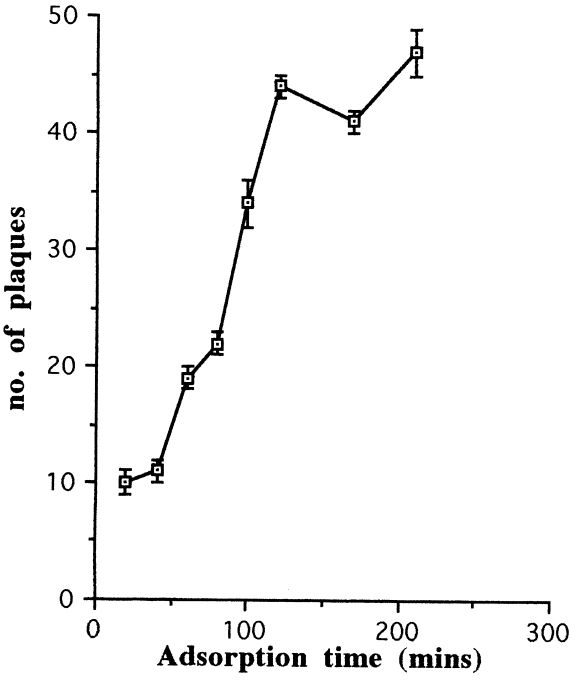

Fig. 2. Graph showing the influence of adsorption time on the subsequent number of rotaviral plaques (10^{-4} dilution).

1.4×10^7 PFU/ml was reduced to 0 PFU/ml after 30 min (upon closer analysis, it was noted that the rate of killing was extremely rapid and the titer had been reduced to 0 PFU/ml at just 10 min or less). Subsequently, the rate of killing at 60°C was investigated; an initial titer of 1.2×10^7 PFU/ml was used in this experiment, and no discernible plaques were detected at the first time point (10 min), which indicates that a temperature of 60°C and above is lethal for rotavirus. The ability of rotavirus to survive slightly lower temperatures was also analysed. The thermal death curve for rotavirus at 50°C is illustrated in Fig. 4 and the associated D' value for this temperature was calculated to be 8 min. The same experiment was performed at 55°C (Fig. 4) which had an associated D' value of 5.8 min.

The ability of UV light to inactivate rotavirus was examined over a range of energies. For each individual assay, the virus sample was exposed and diluted before plaquing. The numbers of remaining infectious viral plaques were then enumerated and compared to the same unexposed virus (control). An increase in UV energy resulted in a greater killing effect. The survival of rotavirus was plotted over the range of energies tested and is presented in Fig. 5.

3.4. Survival and recovery of rotavirus

3.4.1. Fruit juice

An experiment was set up to examine the survival of rotavirus in an artificially contaminated fruit juice

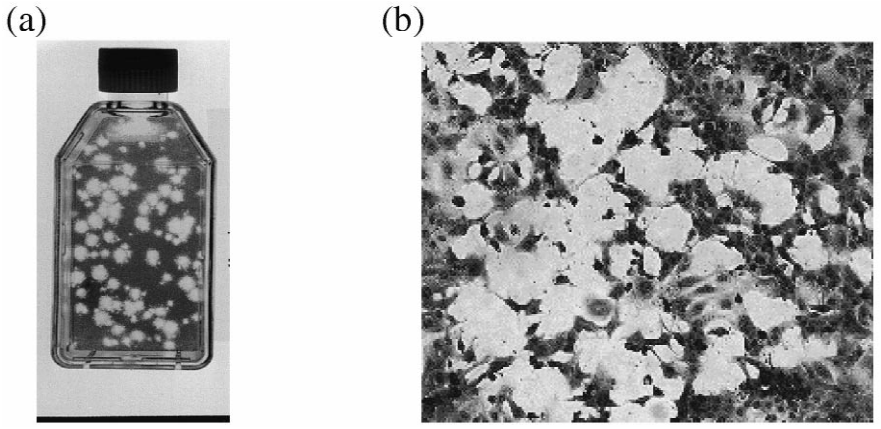

Fig. 3. (a) Photograph which illustrates a flask from a typical plaque assay as described in the Materials and methods section (10^{-5} dilution), which has been stained with 1% crystal violet (temperature of incubation: 37°C). (b) Photograph which illustrates the associated localised cytopathic effect from a single plaque as viewed microscopically (40×).

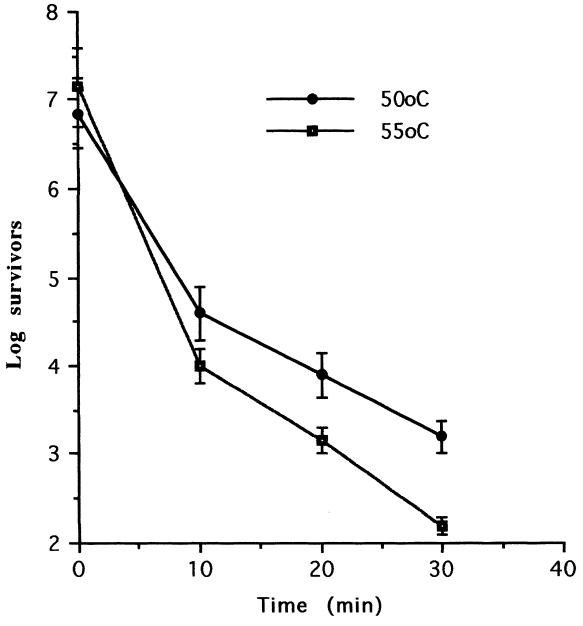

Fig. 4. Graph showing the rate of killing for rotavirus in DMEM at 50 and 55°C as determined by a plaque assay.

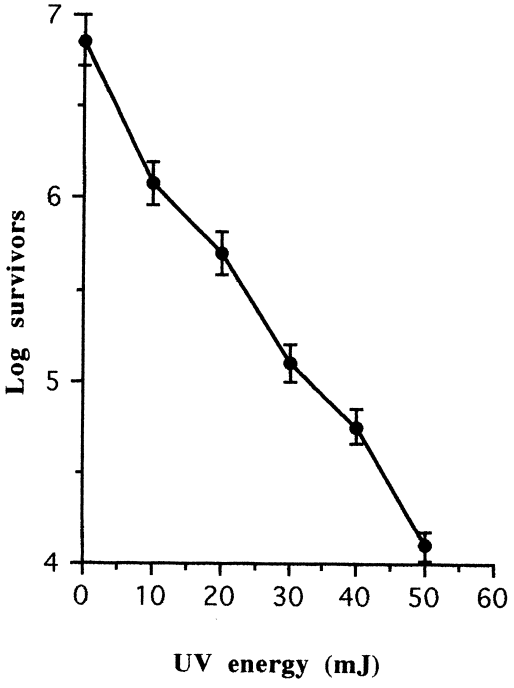

Fig. 5. Graph showing the rate of killing for rotavirus in DMEM following an increase in UV energy (10–50 mJ) (260 nm).

over time. The pH of the juice was 3.01 before filtering and 2.98 afterwards. Following a plaque assay, the initial rotavirus titer used in the experiment was calculated to be 1.3×10^6 PFU/ml, the titer recovered from the juice after 3 days at 4°C was determined to be 1.0×10^6 PFU/ml (77% recovery), and the control titer (in DMEM at 4°C) was 1.1×10^6 PFU/ml (84% survival). This indicates that rotavirus has the potential to survive well at refrigeration temperatures and in low pH fruit juice.

3.4.2. Infant formula milk

The ability to enumerate infectious rotavirus from artificially contaminated infant formula milk was

determined. The initial titer of the virus used in this experiment was 1.2×10^7 PFU/ml, and the number of infectious viruses recovered after the experiment was 3×10^6 PFU/ml (25% of the original titer). This may demonstrate the potential inhibitory characteristics of formula milk or represent an inherent difficulty in recovering culturable virus from a complex medium such as milk.

3.4.3. Lettuce

The optimised plaque assay was also used to recover infectious rotavirus from artificially contaminated lettuce. The initial titer was 5.9×10^6 PFU/ml, and the number recovered following the experiment was 5.5×10^6 PFU/ml (representing a 93% recovery rate). This recovery rate is high and suggests that the plaque assay has a role to play in experiments where it is not only important to enumerate viruses, but also where it is necessary to examine the effect of foods on viral infectivity.

4. Discussion

The development of cell culture and plaque assay techniques has been instrumental in furthering rotavirus research over the last 20 years. In this study we have employed and adapted pre-existing techniques to propagate rotavirus in cell culture and have demonstrated how the presence of infectious rotavirus can be confirmed by analysing tissue culture supernatant for viral RNA. The application of rotaviral RNA analysis to tissue culture supernatants is important for confirming the presence of rotavirus, but can also be used to monitor the progress of an infection over time.

The incorporation of trypsin in tissue culture medium is essential for efficient propagation of rotavirus and was also found to be a critical component of the overlay medium for these plaque assays. Furthermore, we found it necessary to optimise the concentration of FCS in the overlay medium as this greatly affected the number and quality of plaques obtained. The serum may have contributed to stabilising the cell monolayer and our results are in agreement with work previously reported by Imammura et al. (1991). The length of the adsorption time was also shown to be an important factor as we observed a large variation in plaque numbers over a 3.5 h period for the same virus dilution. Burke and Mulcahy (1980) and Fendrick and Hallick (1983) have also reported that the length of this adsorption time greatly affects the number of plaques recovered, although both these studies recommended a 60 min adsorption time.

Even though tragacanth gum has been used in previous viral plaque assays (Jochim and Jones, 1976; Dobos, 1976; Manning and Collins, 1979), there is no record of it being used to enumerate rotavirus. The main advantage of using tragacanth gum is that it can be applied at room temperature and consequently does not result in possible heat-induced damage of cells commonly associated with agar (Dobos, 1976). During these assays we found it to be an effective immobilising agent and, when used in conjunction with the other overlay components, generated discrete countable plaques.

The potential applications of this assay were investigated and we demonstrated how viral numbers could be enumerated using our plaque assay. Due to the fact that rotaviruses have been associated with food and water, we decided to simulate a variety of heating conditions in the laboratory, and evaluated the effects of a range of temperatures on the survival of rotavirus. We showed that temperatures of 50 and 55°C were sufficient to reduce the titre of rotavirus by almost 3 and 5 logs, respectively (in line with results of Estes et al., 1979, who reported over a 2 log decrease in numbers at 50°C). We also demonstrated that rotavirus is unable to survive a simulated pasteurisation cycle of 63°C × 30 min and further analysis revealed that 60°C for 10 min was sufficient to reduce the viral titer by at least 7 logs. From these experiments we have shown that the virus is relatively heat sensitive, and thorough cooking and reheating of potentially contaminated foods should be sufficient to destroy the infectivity of contaminating viruses.

The potential for inactivating rotaviruses using ultra-violet radiation was tested in our laboratory. We found a linear decrease in viral numbers over the range of UV tested and the most effective dose was 50 mJ which was sufficient to reduce the initial viral titer by >2.5 logs. This is similar to the data presented by Chang et al. (1985), who have shown that the most effective range of energy required to inactivate rotavirus by up to 3 logs was between 28 and 42 mJ.

We have also shown that rotavirus is relatively stable at low pH and can survive at refrigeration temperature following experiments with artificially contaminated fruit juice which suggests that the survival of rotavirus at refrigeration temperatures is high (at least over 3 days). We chose acidic fruit juice as many young children consume similar beverages and felt that it may be a potential source of viral transmission. Estes et al. (1979) has shown that rotavirus is relatively stable at pH 3.5 (losing ~20% of its infectivity at this pH) which is similar to our reported results. We showed that the combination of 4°C and a low pH was enough to reduce the initial viral numbers by 26%.

There is reported evidence which incriminates rotavirus as a potential contaminant of foods (Cliver, 1996), particularly foods which have come into contact with carriers of the virus (i.e. faecally borne). Improperly handled foods which do not undergo a cooking stage (e.g., salads) are at risk of harbouring the virus and, as an example, we investigated the ability of rotavirus to survive after being allowed to dry onto lettuce leaves. After 4 h we were able to recover 93% of the original titer, highlighting the potential for rotavirus to be transmitted through foods. We chose lettuce as it is one of the foods which rotavirus has been associated with in the past (Hernandez et al., 1997) and can be easily contaminated.

A large percentage of newborn babies are fed powdered milk and we set out to examine the survival/recovery of rotavirus from commercially available formula milk. During analysis, we recovered 25% of the original titer which shows at least that the virus can survive in, and is recoverable from, formula milk. However, it is difficult to establish whether the large reduction in titer was due to inhibitory compounds in the milk (e.g., apo-lactoferrin; Superti et al., 1997) or attributable to the virus being impeded during filtration.

5. Concluding remarks

We report here an efficient and versatile plaque assay for rotavirus which was optimised and used to enumerate tissue culture adapted viruses. The critical parameters for this assay are the adsorption time and the inclusion of trypsin and FCS. We have also shown that this strategy can easily be adapted to enumerate the virus from contaminated foods, and has been shown to be effective for evaluating the survival of rotavirus following exposure to deleterious agents, e.g. heat and ultra-violet light.

Acknowledgements

This research has been funded, in part, by grant aid under the food sub programme for industrial development administered by the Department of Agriculture and Food and was supported by national and EU funds. We greatly acknowledge the cooperation of Dr. Paul Masendycz, (Melbourne, Australia) who generously donated virus strains used for cell culturing.

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