Effects of Acidification, Bacterial Fermentation, and Temperature on the Survival of Rotavirus in a Model Weaning Food

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ABSTRACT

The effect of acidification, bacterial fermentation, and temperature on the survival of SA-11 rotavirus in model infant weaning foods was investigated. The influence of added organic acids and of bacterial fermentation on rotavirus survival was explicable solely by the pH achieved. The rotavirus was stable at temperatures representative of tropical ambient and at pH values typical of lactic fermented foods (3.8-4.1). Starch gelatinization temperatures were sufficient to inactivate rotavirus rapidly at neutral pH. Thus, cooking would kill virus although recontamination would remain a concern. A similar lethality to that at neutral pH could be observed in acidified media at lower temperatures. Although effective against bacterial pathogens, lactic fermentation of weaning foods confers little protection against rotavirus unless combined with a mild heat treatment such as might be used prior to serving to enhance palatability.

Diarrhoeal disease is a major cause of morbidity and mortality among children in less developed countries; an estimated billion (10\textsuperscript{9}) episodes occur each year and 4.6 million children under 5 die as a result (26). Diarrhoea can occur repeatedly in the same individual, up to 10 times per annum in some cases (3,5,12), and can lead to malnutrition. This, in its turn, predisposes the child to longer and more severe diarrhoeal episodes and other serious infections (8).

Weaning is a particularly hazardous time for the infant involving loss or dilution of the anti-infective properties of maternal breast milk, its replacement with foods often of low energy density, and the exposure of an immature immune system to new sources of infection in the environment (17). Poor hygienic practices, such as postcooking contamination, and the use of contaminated water in the preparation of weaning foods are often implicated in weaning diarrhoea, and it has been estimated that 15-70\% of all diarrhoea episodes in young children is food associated (10).

A range of pathogens has been associated with weaning diarrhoea including enterotoxigenic and enteropathogenic Escherichia coli, Campylobacter jejuni, Shigella, and rotavirus (3,5). Weaning foods have been found to contain enteric and pathogenic bacteria (2,4,7,14,20), but their role in the transmission of rotavirus is less clear. This is largely due to the difficulty of demonstrating the presence of low numbers of virus in foods (1). However, evidence of the fecal/oral transmission of bacterial pathogens via food, the presence of up to 10\textsuperscript{10} rotavirions per gram in an infected individual’s feces, and the association of rotavirus transmission with water (16,27), all suggest that foodborne transmission of rotavirus can also occur.

Fermentation with lactic acid bacteria is an established technique whereby spoilage and pathogenic bacteria can be inhibited. Inhibition is the result of a combination of factors, the most important of which are a decrease in pH and the production of organic acids (15).

Studies on the use of lactic fermentation to improve the safety of weaning foods have yielded promising results. Using prefermented formulations with a pH below 4, significant lethal effects on a range of bacterial pathogens have been demonstrated (18,21,22). In a Ghanaian field trial, fermentation was shown to be an effective means of reducing the levels of gram-negative bacteria in weaning foods (19).

There is, however, no published information on the effect of lactic fermentation on the survival of foodborne viruses in weaning foods. Slight lethality towards a coliphage has been reported (22), but the significance of this with regard to diarrhoeagenic viruses is not known. More direct studies using the pathogenic viruses themselves are hindered by the difficulties of cultivation in vitro. The simian rotavirus offers some advantages in this respect: it grows well in cell culture, causes diarrhoea in a fellow primate, and has many features in common with human rotavirus (25,28). It may also serve as a model for other nonculturable enteric viruses. Here we report a study on the effects of heating, acidification, and fermentation on the survival of simian rotavirus SA-11 in a model infant weaning food.

MATERIALS AND METHODS

Virus

Simian rotavirus SA-11 was supplied by the School of Biological Sciences, University of Surrey. It was purified five times by limiting dilution and inoculation onto MA-104 cells and examined by electron microscopy to confirm its identity.

Cells

Cells used were of the MA-104 line of embryo monkey kidney (Burroughs Wellcome, Research Triangle Park, NC) between passages 116 and 152.

Cells were grown in modified medium 199 (Flow Laboratories, McLean, VA) containing 5% fetal bovine serum (FBS), 3%
For viral growth in plastic tissue culture flasks (175 cm²), main­
tenance medium was as above but contained only 2% FBS. For viral growth in plastic tissue culture flasks, the maintenance medium was used with the FBS omitted and the addition of distilled water were made in 100 ml volumes and autoclaved (10 lbs, 121°C, 15 min, St. Louis, MO). To produce virus for incorporation into bacterial fermentation, antibiotics were omitted from the growth medium.

**Bacteria**

*Lactobacillus plantarum,* originally isolated from a commercial meat starter culture, was obtained from the School of Biological Sciences culture collection. It was grown in MRS medium (Oxoid, London) used either as a broth or agar incubated aerobi­cally at 30°C overnight.

**Preparation of model weaning food**

Long-grained, husked, American rice was coarsely ground in a pestle and mortar. Suspensions of 5% and 10% ground rice in distilled water were made in 100 ml volumes and autoclaved (10 psig/10 min). In some cases autoclaved gruel was predigested by the addition of alpha-amylase (Sigma), 500 units/100 ml of gruel, or diastatic malt extract (200 L, ABM Chemicals, Stockport, UK), 1 ml of a 50% solution/100 ml of gruel, and leaving for 5 h at room temperature.

**Virus titration**

Decimal serial dilutions of the sample in virus titration medium were inoculated (50 µl) into four replicate wells of a microtiter plate containing confluent MA-104 cell monolayers. The first well and the last three wells of each row were overlayed microscopically from day 3 of incubation and the titration result being read by cytopathic effect (CPE) on the final day. Wells were left at room temperature for 30 min to allow virus adsorption. A further 200 µl of virus titration medium was added to each well, the plates sealed with selfadhesive plastic sheet, and incubated at 37°C for 6 d in a conventional incubator. Wells were inspected microscopically from day 3 of incubation and the titration result noted. The virus titers were expressed as the number of infective units (i.e., virus titer by a factor of approximately 10).

**Heat inactivation of virus**

Suspensions of virus (0.5 ml) were added to a number of capped empty glass bottles (5 ml) pre-equilibrated in a water bath at the designated temperature. Exposure time was taken from the time of addition. At each sampling, 0.4 ml was taken from a bottle and transferred to a similar bottle precooled in ice and stored at 4°C before titration. Where virus was suspended in starch solutions, the suspension was incubated overnight at 22°C with alpha-amylase (Sigma) at 500 units/ml sample after treatment and before titration. This procedure increased the apparent virus titer by a factor of approximately 10.

**Acid inactivation of virus**

Virus suspension in viral growth medium in a glass bijou was adjusted to the required pH by the addition of a predetermined volume of acetic or lactic acid followed by immediate vortex mixing prior to heat treatment as above. Samples (0.1 ml) were removed periodically and added to virus titration buffer (0.9 ml) at room temperature (22°C), which served both to cool and neutralize the sample, and stored at 4°C before titration.

**RESULTS**

The effect of heating SA-ll rotavirus in viral growth medium is shown in Fig. 1. Plotting both elapsed time and virus titer on logarithmic scales allows sampling times from 1 min to 24 h to be incorporated sensibly on the graph.

At 30 and 40°C, the virus was stable for at least 24 h. At 50°C, the decrease in titer was not constant; it fell by 1-log cycle in 20 min and by 1.5-log cycles after 1 h. The apparent decrease in death rate with time is probably not due to a subpopulation of more heat resistant virus since the virus was purified by limiting dilution to ensure a homogeneous population. More probably, this effect is due to the presence of some virus clumps, an established feature of rotavirus (23). These would require multiple inactivation to eliminate a single infective unit and give the appearance of a more resistant subpopulation. At higher temperatures, virus inactivation was rapid with a 3.25-log cycle reduction at 60°C in 5 min and a 4.5-log cycle reduction in 1 min at 70°C.

No protective effect from food materials was apparent when SA-ll was heated at 60°C for 5 min. The same log reduction in titer (3.25) was obtained with viral growth medium and rice gruels containing 5 and 10% rice.

The reduction, relative to untreated controls, in the titer of rotavirus incubated at different temperatures and at pH values typical of the range obtained by lactic fermentation (3.3 and 4.0) is illustrated in Fig. 2. At all temperatures the virus was more heat sensitive than at neutral pH (Fig. 1), and was most sensitive at the lower pH value. At 30°C, the virus was relatively stable, the titer decreasing by 0.25- and 1.00-log cycles at pH 4.0 and 3.3, respectively, in 24 h while log reductions of 0.5 (pH 4.0) and 2.25 (pH 3.3) were obtained in 1 h at 40°C and 2.0 (pH 4.0) and 2.75 (pH 3.3) at 50°C in 5 min. In the experiments described, DL lactic acid was used as acidulant; similar results were obtained when acetic acid was used (results not shown).

Exposure of rotavirus to a culture filtrate of *L. plantarum* (MRS broth, pH 3.5) at 30°C produced similar results to artificially acidified viral growth medium with a log reduction of 0.5 unit after 23 h. Results from the inclusion of rotavirus in actively growing cultures of *L. plantarum* in
MRS broth and a model weaning food are presented in Table 1. Rice was used in the model weaning food since maize-based porridge showed toxicity to the MA-104 cells used for rotavirus enumeration. A lower pH was attained in MRS broth (3.0-3.2) after 24 h at 30°C than in the weaning food (3.8-3.9). No marked change occurred in virus titer in either medium. Incubation for a further 24 h produced little or no further decrease in pH or in the virus titer of the weaning food but gave a 1.5-log decrease in virus titer of the MRS broth. Heating prefermented weaning food for 24 h at 40°C or 5 min at 50°C produced a log reduction comparable to that produced by heating in artificially acidified medium.

TABLE 1. Effect of bacterial fermentation with \( L. \) \( \text{plantarum} \) and subsequent heat processing on survival of SA-11 rotavirus in MRS broth and rice gruel.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Titer reduction (log_{10})</th>
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<tbody>
<tr>
<td>MRS broth(^1)</td>
<td>further 24 h at 30°C 1.5 (pH 3.1)</td>
</tr>
<tr>
<td>Rice gruel(^2)</td>
<td>further 24 h at 30°C 0.25 (pH 3.8)</td>
</tr>
<tr>
<td></td>
<td>further 24 h at 40°C 2.75 (pH 3.9)</td>
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<tr>
<td></td>
<td>further 5 min at 50°C 3.25 (pH 3.8)</td>
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</tbody>
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\(^1\) Inoculated with \( L. \) \( \text{plantarum} \), incubated at 30°C for 24 h (log titer reduction 0.25, final pH 3.2).

\(^2\) 10\% w/v rice, pretreated with amylase, inoculated with \( L. \) \( \text{plantarum} \) and incubated at 30°C for 24 h (log titer reduction 0.25 - 0.25, final pH 3.8-3.9).

**DISCUSSION**

The degree to which lactic fermentation of weaning foods can control rotavirus transmission depends critically on how fermentation is incorporated into existing food preparation practices and at what stage rotaviral contamination is introduced. When heated at near neutral pH, the rotavirus was very rapidly degraded at typical starch gelatinization temperatures (e.g., 60°C), and this effect was not mitigated by the presence of food materials.

Other workers have found no protective effect from milk on survival of bovine rotavirus over 2 h at 37°C (24), but differences between the heat stability of rotavirus in much more dilute media such as tris buffer and water have been reported (11).

Rotavirus displays markedly greater stability than bacterial pathogens at pH values typical of lactic fermented foods. At 30°C, representative of tropical ambient temperature, the rotavirus titer declined by only 0.25-log cycle in 24 h at pH 4.0. Work with bacterial pathogens has shown typical decreases of around 4-log cycles in less than 6 h at pH 3.8 (22).

It is well established that, at the same pH, acetic acid has a more pronounced antibacterial effect than lactic acid (9). Here, both acids displayed similar activity against rotavirus. This is consistent with, and extends, the findings of Weiss and Clark (28) and Rodger et al. (25) who found antiviral effects to be related to pH and not the acidulant used. This is due to differences in the mode of action of weak organic acids against bacteria and viruses. In bacteria, activity depends not only on pH but on the ability of the undissociated acid to penetrate the bacterial plasma membrane and disrupt intracellular processes (6). This does not apply in a nonenveloped virus such as rotavirus which is probably inactivated by denaturation of capsid proteins by low pH (25).

The results obtained when rotavirus was incubated in the presence of an active culture of \( L. \) \( \text{plantarum} \) and culture filtrates do not indicate the presence of any antiviral metabolites other than organic acid.

The increased lethality when rotavirus was heated at low pH suggests that the protective effect of fermentation can be enhanced considerably if a prefermented weaning food is reheated prior to consumption.

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**REFERENCES**

ROTAVIRUS SURVIVAL IN WEANING FOODS


