Comparison of Methods for the Recovery of Virus Inoculated into Ground Beef

JOHN T. TIERNEY, ROBERT SULLIVAN, EDWARD P. LARKIN, AND JAMES T. PEELER

Bureau of Foods, Food and Drug Administration, Cincinnati, Ohio 45226

Various methods for the recovery of virus inoculated into ground beef were investigated in an attempt to develop a sensitive system that could be used to detect viral contaminants in market foods. A 100-g sample, inoculated with poliovirus 1, was suspended in 150 to 900 ml of Eagle minimum essential medium, pH 8.5, and mixed in either plastic bags or plastic cups on a mechanical shaker. The particulate materials were removed by means of cheese cloth, glass wool, woven fiber glass, or low-speed centrifugation. Large volumes of fluid were concentrated by ultrafiltration. Microbiological contamination was controlled by high antibiotic concentrations or by filtration. Quantitative plaque-forming-unit recovery of the virus was determined by utilizing an agar overlay technique on Vero cell cultures. The data indicated that from 20 to 50% of the seeded virus could be recovered from a 100-g sample of ground beef. The glass wool and woven fiber glass methods were the most effective, with recovery of approximately 50% of the inoculated virus.

In 1970, we reported the isolation of viruses from 3 of 12 market samples of ground beef. The viruses were identified as polioviruses 1, 2, and 3, and echovirus 6 (10).

Approximately 50% of the beef in the United States is consumed in the form of ground beef or hamburger (6). The eating habits of our population are such that much of this beef is consumed in a rare to medium-rare state. The finding of viruses in a common food that is handled by both the processor and the consumer before cooking and is eaten in a semicooked condition indicated that this food could be of public health significance. Possibly such a food could be the carrier of agents in food-borne outbreaks of unknown etiology (2, 3). The transport of viruses by foods may occur as frequently as meal time (1, 2, 4, 8).

A study was initiated to develop effective methods for the recovery of viruses from ground beef—methods that could be used in laboratories in much the same way as those used to test for bacterial content. In the study reported previously, 5-g samples of ground beef were analyzed. The small number of viruses found in the samples indicated that the probability of viral recovery would be better if a larger sample were examined. Therefore, in this study, methods were developed for the recovery of viruses from 100-g samples of ground beef. It was anticipated that such methods could also be applied to the recovery of viruses from other foods.

MATERIALS AND METHODS

Ground beef. Fresh ground beef was purchased from local retail markets on the same day that the samples were run. Samples (100 g) were weighed into sterile plastic bags, and 1 ml of virus suspension was added to the ground beef. The bags were sealed, and the contents were kneaded by hand for approximately 5 min. The previous study on the homogeneity and distribution of the virus in the ground beef indicated that this was an effective method for distributing the virus throughout the sample (10).

Tissue culture. Vero monkey kidney cell cultures (ATCC, CCL#81) passage 125 were used as the source of tissue culture preparations. The culture was propagaged in 6-oz (170.1-g) prescription bottles; the confluent cell sheets were trypsinized, split 1 to 8, and recultured in sufficient quantities for viral growth studies.

Growth medium. The growth medium used for tissue culture was a 1:1 mixture of Leibovitz medium (L-15) and Eagle minimum essential medium (MEM) with Hanks salts containing 10% fetal bovine serum and 0.075% $NaHCO_3$. The medium provided excellent growth and maintained the cells for 14 days without having to be changed.

Plaque assay system. A previously reported viral plaque assay system was used (11). An agar medium overlay was used with monolayer (45 cm^2) Vero cell cultures in 6-oz prescription bottles.

Virus. Poliovirus 1 (Mahoney) was passaged three times in primary cell cultures of *Cercopithicus aethiops* (African Green) monkey kidney cells. The cells were freeze-thawed three times, the debris was removed by centrifugation, and 1.2 ml of the virus suspension was placed into each of a number of 2-ml borosilicate glass ampoules and stored at –60 C.

High-antibiotic MEM (HAMEM). MEM with non-essential amino acids in Hanks salts containing 2% fetal bovine serum, 2.5 mg $MgCl_2 \cdot 6H_2O$ per ml (12), and 100 μg of diethylaminoethyl-dextran sulfate (2×10^6 molecular weight) per ml was used for the elution of viruses from the ground beef. Antibiotics were added in the following concentrations per ml: 4,740 U of penicillin G, 5,000 μg of streptomycin sulfate, 250 μg of tetracycline hydrochloride, and 5.0 μg of amphotericin B. One normal NaOH was added to raise the pH to 8.5 for elution of the virus and also to prevent coagulation of the meat slurry (10).

Glass wool and woven fiber glass methods. The methods using glass wool and woven fiber glass are similar and are described jointly. A 100-g sample of virus-containing ground beef and 100 ml of HAMEM were placed in a plastic bag. The bag and contents were shaken vigorously by hand, and the pH of the slurry was readjusted to 8.5. The plastic bag containing the slurry was placed on a mechanical shaker and shaken for 15 min. The contents were poured through 4 g of either glass wool or woven fiber glass, which had been placed in a funnel. The wool or fiber glass had been pretreated with 20 ml of HAMEM. The bag was rinsed with 30 ml of the HAMEM, and the rinse was added to the funnel. Approximately 100 ml of clarified meat slurry can be obtained after 1 h. A laminar vertical flow cabinet was used to prevent contamination of the sample during processing. The total recovered fluid was inoculated into 30 bottles of Vero cell monolayers; the bottles were incubated for 2 h at 36 C, and the Vero cell monolayers were then overlayed with agar medium. After the agar solidified, the bottles were inverted and incubated at 36 C. The plaques were counted and marked daily for 14 days.

Potato ricer method. The preliminary processing of the 100-g sample was the same as that outlined for the glass wool and fiber glass methods. After being shaken for 15 min, the contents of the bag were poured into a commercial stainless-steel potato ricer. (The potato ricer is a hand-operated apparatus used to compress cooked potatoes through small holes in a metal container.) In this process the potato ricer was lined with four layers of cheese cloth pretreated with 20 ml of HAMEM. The bag was rinsed with 30 ml of the HAMEM; this fluid was added to the potato ricer. The liquid was squeezed from the meat-fluid mixture by pressure. The total recovered extract (100 to 110 ml) was inoculated onto 30 Vero cell monolayers and incubated for 2 h at 36 C. The cultures were processed as described above.

Low-speed centrifugation method. The ground beef sample was placed in an 8-oz (226.8-g) plastic cup having a tight-fitting lid, and 100 ml of HAMEM was added. The cup was shaken vigorously by hand, the pH of the slurry was readjusted to 8.5, and the sample was then mixed on a mechanical shaker for 20 min. The pH of the slurry was readjusted to 8.5, and the sample was centrifuged for 20 min at 690 × *g* in a preparative centrifuge. After centrifugation, the supernatant fluid was decanted. The pellet was resuspended in 50 ml of HAMEM, shaken, and centrifuged a second time. The supernatant fluid was removed and combined with the first supernatant. The total fluid volume, approximately 110 to 120 ml, was inoculated onto 30 Vero cell monolayers, and the bottles were processed as described for the other methods.

Ultrafiltration method. In previous studies with 1- and 5-g samples, a meat and liquid ratio of 1:10 resulted in the extraction and recovery of a high percentage of virus from the ground beef. To simulate this study with a larger ground beef sample, 800 ml of HAMEM and a 100-g inoculated beef sample were placed in a plastic bag. The sample was mixed on a shaker for 15 min. The contents were poured into a potato ricer containing four layers of cheese cloth pretreated with 50 ml of HAMEM. The plastic bag was rinsed with 50 ml of HAMEM, and the fluid was poured into the potato ricer. The fluid was removed by pressure. Ten grams of diatomaceous earth (Celite 545) was added to the clarified slurry, and the fluid was filtered to remove bacterial contaminants and particulate material that would clog the 0.0075-μm filter used to concentrate the virus. Three types of filters—(i) 0.45-μm cellulose acetate (Gelman Co.), (ii) 0.40-μm polycarbonate ("Nuclepore," General Electric Co.), and (iii) 0.45-μm silver (Selas Flotronics Co.)—were used. The filters were pretreated with fetal bovine serum immediately before use. It required from ½ to 8 h to process the sample, depending on the type of filter used.

Each filtrate was concentrated by ultrafiltration with a protein-enrichment membrane (PEM) of 0.0075-μm porosity (Gelman Co.) at 7 C. This process required from 8 to 16 h. The virus was eluted from the membrane with 60 ml of fetal bovine serum (9).

Samples (10 ml) each were taken after passage through the filter and from the material eluted from the PEM. Dilutions (10-fold) were made, and 1 ml was inoculated into each of five bottles of Vero cell monolayers. Agar medium overlay, incubation, and the procedures for counting were the same as described before.

RESULTS

Two groups of experiments were done to compare various methods used to extract poliovirus from ground beef. In the first group, glass wool, woven fiber glass, the potato ricer, and low-speed centrifugation methods were evaluated for effectiveness in the clarification of a ground beef slurry and effectiveness of viral recovery. All the methods produced clarified suspensions suitable for inoculation onto cell sheets for viral plaque-forming units (PFU) enumeration. The results are shown in Table 1. Viral recovery data for the glass wool or woven fiber glass methods were similar, with mean

recoveries of 48 and 49%, respectively. The potato ricer method, in addition to being quite cumbersome in application, produced the lowest viral recovery (19%) of the four methods analyzed.

The glass wool, woven fiber glass, and potato ricer samples were processed in a laminar flow cabinet to prevent contamination of the product during handling. If adequate aseptic procedures are followed and the sample is covered with sterile aluminum foil, it is possible to process samples without a protective cabinet. However, when only one or two plaques are recovered, it may be difficult to prove that the virus came from the sample and was not a result of airborne contamination. The low-speed centrifugation method was developed in which the sample was processed in a relatively closed system. A 33% viral recovery resulted with this method. The four methods are compared in Table 2.

In the second group of experiments, a comparison of the three bacterial retaining filters used as prefilters in the viral concentration study indicates that the use of the polycarbon-

TABLE 1. *Recovery of virus after passage of inoculated ground beef slurries through glass wool, woven fiber glass, a potato ricer, or after low-speed centrifugation*

Method	Input (total PFU)[a]	Recovery (total PFU)	Recovery (%)
Glass wool	180	82	45.5
	180	100	55.6
	230	95	41.3
	230	110	47.8
Woven fiber glass	200	90	45.0
	200	102	51.0
	200	119	59.5
	370	146	39.5
	370	178	48.1
	370	188	50.8
Potato ricer	475	101	21.3
	475	94	19.8
	475	91	19.2
	475	93	19.6
	475	72	15.2
Low-speed centrifugation	120	37	30.8
	120	45	37.5
	120	46	38.3
	120	48	40.0
	120	59	49.2
	300	52	17.3
	300	72	24.0
	300	85	28.3
	300	87	29.0

[a] PFU, Viral plaque-forming units.

TABLE 2. *Comparison of methods used for the recovery of poliovirus from inoculated ground beef*

Source	Mean % recovery	% Coefficient of variation	No. of observations
Potato ricer filter	19.0	12.0	5
Low-speed centrifugation	32.7	29.2	9
Glass wool filter	47.6	12.6	4
Woven fiber glass filter	49.0	13.7	6

ate filter resulted in faster processing (30 to 60 min) as compared to the silver filter (3 h) and the cellulose acetate filter (8 h). About 37% of the virus was retained on the cellulose acetate filter, 24% was retained by the polycarbonate filter, and 11% was retained by the silver filter. The time required to concentrate the fluids on the ultrafilter was less than 8 h when the cellulose acetate prefilter was used, 16 h for the polycarbonate filter, and 20 to 24 h for the silver filter. Some bacteria and molds passed through the silver filter. Results of this study are presented in Tables 3 and 4. Clogging of the filters was reduced by preliminary clarification through cheese cloth and by the use of diatomaceous earth as a filter aid.

In Tables 2 and 4, the mean recoveries and percent coefficient of variation were computed for each of the combinations described above. The percent coefficient of variation is defined as 100 X (standard deviation/mean recovery). A value of 20 to 25% might be considered good for the present virus work. A recovery of 50% of the initial virus load is considered adequate, considering the small viral inoculum for a 100-g sample.

In the first series of experiments, the glass wool and woven fiber glass methods met the above criteria and perhaps merit further testing. The methods used in the second phase of the study were also satisfactory. Although the mean recoveries differ in the concentration step, the variation is large, as shown in Table 4. The distribution of the percent recoveries is unknown; however, it is assumed that the means are normally distributed. The following F test was performed to examine the null hypothesis that the three mean recoveries were equal. The test is performed at the α = 0.01 level. F = variation between groups/variation within groups. $F_{2, 19}$ = 0.04318/0.01747 = 2.47. The value for F for these is 2.47. Since this value is less than the critical value (5.93), it is assumed that the mean recoveries could not be shown to differ.

DISCUSSION

In earlier studies, the addition of the ground beef slurry directly to the cell culture resulted in the mechanical stripping of the cell sheet. Various clarifying materials and methods were studied in an attempt to eliminate the particulate material. These investigations culminated in the development of the glass wool, woven fiber glass, potato ricer, and low-speed centrifugation methods. In all these methods, a high-antibiotic medium, pH 8.5, was used to elute the virus from the ground beef and to control bacterial contaminants.

The viral recovery data indicate that the glass wool and woven fiber glass methods both give recoveries of approximately 50%. The use of the low-speed centrifugation method resulted in recovery of 33% of the inoculated virus. All the methods appear to be effective for the recovery of virus from a 100-g sample of ground beef.

A comparison of the three bacteria-retaining filters used in the viral concentration study indicates that the 0.45-μm silver filter allowed for more effective passage of the virus (Table 4). These data confirm results reported by Hahn et al. (7). Hahn also demonstrated the inability of the silver filters to retain 100% of *Serratia marcescens*. It is possible that bacteria and particles of ground beef passed through the filter and interfered with recovery efficiency on the ultrafilter.

Cliver (5) reported that treatment of the filter with serum before filtration greatly improved the filtration of the virus. Probably the 2% serum in the HAMEM plus the serum protein in the meat aided filtration. Hahn et al. (7) stated that the filterability was greatly enhanced by the protein extracts in the medium.

If the concentration of virus in the sample is of such low magnitude that virus may be missed unless the total fluid volume is analyzed, concentration methods are of value. From the viewpoint of economics, the use of 4 or 5 culture bottles, as compared to 30, has a distinct advantage. However, the initial cost of the filter holders and the additional time required in the processing of the sample must be considered when selecting a method.

TABLE 3. *Viral recovery from ground beef after filtration and concentration[a]*

Input (total PFU)[b]	Filtrate (total PFU)	% Recovered	Concentrate (total PFU)	% Recovered
Cellulose triacetate (Gelman)				
5,000	3,150	63.0	2,400	48.0
8,000	3,150	39.4	2,400	30.0
11,900	12,155	102.1	7,500	63.0
20,000	12,550	62.8	10,500	52.5
37,000	10,400	28.1	8,300	22.4
37,000	14,900	40.3	12,000	32.4
37,000	18,100	48.9	16,750	45.3
37,000	43,900	118.6	14,250	38.5
		62.9[c]		41.5[c]
Silver (Selas)				
300	280	93.3	150	50.0
1,900	2,480	130.5	1,100	57.9
4,830	3,150	65.2	2,400	49.7
4,830	NT[d]	NT	3,600	74.5
5,200	5,988	115.1	3,720	71.5
8,000	3,600	45.0	2,400	30.0
		89.8[c]		55.6[c]
Polycarbonate (Nuclepore)				
1,025	700	68.3	360	35.1
5,000	3,900	78.0	1,320	26.4
6,560	6,480	98.7	3,000	45.7
8,000	7,760	97.0	4,350	54.4
8,000	6,940	86.8	4,200	52.5
8,000	7,144	89.3	4,000	50.0
8,750	2,900	33.1	3,000	34.3
9,400	5,605	59.6	3,200	34.0
		76.4[c]		41.6[c]

[a] Protein enrichment membrane, porosity 0.0075 μm (Gelman).
[b] PFU, Viral plaque-forming units.
[c] Mean.
[d] NT, Not tested.

TABLE 4. *Statistical evaluation of viral recovery in the filtrate by using three filters and in the concentrate after each filter*

Source	Filtration step		Concentration step		No. of observations
	Mean ± SD	% Coefficient of variation	Mean ± SD	% Coefficient of variation	
Gelman filter, 0.45 μm	62.9 ± 31.9	50.7	41.5 ± 13.2	31.9	8
Silver filter, 0.45 μm	89.8 ± 35.1	39.0	55.6 ± 16.4	29.4	6
Nuclepore filter, 0.40 μm	76.4 ± 22.1	29.0	41.6 ± 10.2	24.6	8

LITERATURE CITED

1. Aycock, W. 1927. A milk-borne epidemic of poliomyelitis. Amer. J. Hyg. 7:791-803.
2. Berg, G. 1964. The food vehicle in virus transmission. Health Lab. Sci. 1:51-59.
3. Cliver, D. O. 1966. Implications of foodborne infectious hepatitis. Health Rep. Pub. Health Serv. 81:159-165.
4. Cliver, D. O. 1967. Food associated viruses. J. Health Lab. Sci. 4:213-221.
5. Cliver, D. O. 1968. Virus interaction with membrane filters. Biotechnol. Bioeng. 10:877-889.
6. Consumer Reports. Aug. 1971. A close look at hamburger, p. 478-483, vol. 36.
7. Hahn, R. G., J. B. Hatlen, and G. E. Kenny. 1970. Comparative poliovirus permeability of silver, polycarbonate, and cellulose membrane filters. Appl. Microbiol. 19:317-320.
8. Lipari, M. 1951. A milk-borne poliomyelitis episode. N.Y. State J. Med. 51:362-369.
9. Rao, V. C., R. Sullivan, R. B. Read, Jr., and N. A. Clarke. 1968. A simple method for concentrating and detecting viruses in water. J. Amer. Water Works Ass. 60(11):1288.
10. Sullivan, R., A. C. Fassolitis, and R. B. Read, Jr. 1970. Method for isolating viruses from ground beef. J. Food Sci. 35:624-626.
11. Sullivan, R., and R. B. Read, Jr. 1968. Method for recovery of viruses from milk and milk products. J. Dairy Sci. 51:1748-1751.
12. Wallis, C., and J. L. Melnick. 1962. Magnesium chloride enhancement of cell susceptibility to poliovirus. Virology 16:122.