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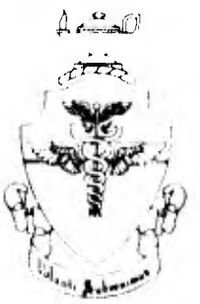

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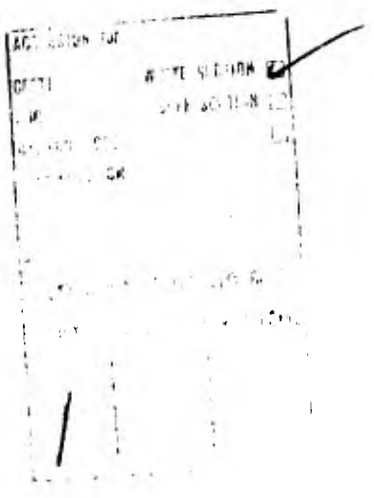

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FOREWORD

The research reported in this paper was performed at the Food Research Institute of the University of Wisconsin, Madison, Wis., under contract No. AF 41(609)-2982 and task No. 775803. Dr. Dean O. Cliver served as principal investigator. John E. Herrmann, research assistant, innovated and performed much of the experimental work and Miss Rose M. Wagner produced all of the tissue cultures used in the experiments.

The monitoring agency was the Physiology Branch, USAF School of Aerospace Medicine. The contract monitor at the start of the contract was Major Norman D. Heidelbaugh. At the close of the contract the monitor was Major William T. Ashby.

The work was accomplished during the period 1 August 1966 through 30 June 1967, and the report was received for publication on 7 December 1967.

The animals involved in this study were maintained in accordance with the "Guide for Laboratory Animal Facilities and Care" as published by the National Academy of Sciences-National Research Council.

This report has been reviewed and is approved.

GEORGE E. SCHAFER
Colonel, USAF, MC
Commander

ABSTRACT

Methods developed for detection and quantitation of food-borne virus are described. Twenty-five-gm. samples of cottage cheese, contaminated with various quantities of Coxsackie virus, type A9, comprised the model system. Two of the methods presented have at least a 50% probability of detecting virus at levels below 5 plaque-forming units/25-gm. sample. Noteworthy aspects of these methods include use of a glycine-NaOH buffer (pH 8.8) containing approximately molar $MgCl_2$ as the diluent in which the sample is slurried, treatment of the slurry with Freon TF and bentonite to facilitate centrifuge clarification, and concentration of the clarified sample extract by a 2-stage process employing polyethylene glycol followed by ultracentrifugation. Virus in the final concentrate (0.5 ml.) of the sample has been detected and quantitated by the plaque technic in rhesus monkey kidney cell cultures. Time elapsed in processing the sample approaches 2 days, and the inoculated cultures may have to be observed for as long as 7 days thereafter. If these levels of sensitivity are desired, and if 12 samples per day are tested on a routine basis, the cost savings achieved by employing these methods rather than testing sample extracts without concentration may range from 75% to 90%.

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I. INTRODUCTION

Enough outbreaks of human disease are now on record to suggest that food is occasionally a vehicle in the transmission of virus (1). This epidemiologic record has been taken to indicate that, for viruses infecting the gastrointestinal tract of the human food handler, mishandling of foods occasionally occurs, with resultant contamination of the product with virus-containing feces. Evidence indicates that even a thoroughly cooked food may be rendered unsafe if it is mishandled by an infected individual before packaging or just before serving.

There is a clear need for sensitive methods of detecting food-borne viruses. Such methods could be employed in epidemiologic investigations, in research on the inactivation of food-borne viruses, and ultimately in surveillance for virus safety of food products. At present the costs of available methods, including the one to be described in the present report, are such that they would be useful in routine surveillance only under circumstances where the consequences of producing a virus infection as a result of food contamination would potentially be so dire as to outweigh the expense of the testing.

II. MODEL SYSTEM

This study describes a model method for the detection and quantitation of low levels of virus infectivity in a food. The selected model system consists of cottage cheese and a laboratory strain of Coxsackie virus, type A9.

Three classes of food might occasionally have to be tested for the presence of viruses: fluids, solids likely to be contaminated in depth, and solids in which contamination is likely to be limited to the surface of the food. Cottage cheese falls into the second of these categories. As such, it seems to present the greatest potential problems in processing for virus detection. Fluid foods are intrinsically in a form which will permit the initiation of testing without further dilution. In the case of surface-contaminated foods, if virus can be dislodged from the surface at all, it can probably be accomplished without adding very much of the substance of the food to the virus suspension. Since cottage cheese lacks form, it must be supposed that, if contaminated, it would be contaminated in depth. It must be converted to fluid form for testing, and the fluid suspension thus obtained will necessarily contain a large quantity of food solids. Much of the solid matter in cottage cheese consists of colloidal protein which can, under certain circumstances, greatly complicate the detection process. Cottage cheese was, therefore, selected as the model food vehicle in these studies because it was potentially an extremely challenging substance.

Coxsackie virus, type A9, was selected as the model virus agent because it is a representative of the smaller human enteric viruses which might be expected to contaminate foods under the circumstances described above. The small size of the particles of this virus tends to complicate the concentration process which is employed in the course of detection. In other respects, this virus is quite tractable: it is relatively heat-stable, and readily detected and quantitated in tissue culture.

III. DETECTION SYSTEM

It was intended that the detection system, as developed, permit the detection of viruses at

extremely low levels of food contamination. Sensitivity of the detection system is both required and limited by the fact that virus multiplies only in appropriate living host cells. Sensitivity is required because there can never be any more virus in a food than was introduced at the time of contamination. Storage of the sample before testing may result in a reduction of virus content, but never an increase; and, since the oral infectious dose for any virus has yet to be established, one must suppose that the presence of *any* infectious virus in the sample is of significance to human health. Sensitivity of detection methods is limited by the inability of viruses to multiply outside living cells because there is, for viruses, nothing comparable to the enrichment technics used in detecting food-borne bacterial pathogens. It is stated explicitly that detection of a virus in the present context means the production by the agent of a demonstrable infection in a living host system. Where the level of virus contaminant is marginal, no alternative exists, in increasing the sensitivity of the test, than to increase the quantity of food sample employed in the testing. From the standpoint of quantitative efficiency of virus recovery, the most efficient means of achieving an increase in sensitivity then would be to increase the sample size, prepare all of it in fluid form as discussed above, and inoculate it into the requisite quantity of living host substrate. The cost of the host system, however, places severe limitations upon this approach. The alternative employed in the present study is to reduce the sample volume by concentration, so as to limit the quantity of living substrate required to test a given quantity of food. Tissue cultures comprise the host system employed in detection in the present study, so it can be stated that the reduction of sample volume by concentration is warranted only if the cost of the concentration procedure, and of the necessary additional preparation of the sample suspension before concentration, is less than the alternative cost of the additional tissue cultures which would be consumed in testing the entire volume of the original sample suspension, allowing for the additional virus losses which may occur during preparation and concentration.

IV. SAMPLE PROCESSING

It has been shown that fluid can be removed from dilute virus suspensions through dialysis tubing by the hydrophilic action of polyethylene glycol (2, 5). The advantages of the polyethylene glycol method are relatively low cost per sample and virtually unlimited sample capacity, while the principal disadvantage is relatively low quantitative efficiency of enterovirus recovery, particularly when the method is used for total concentration (2). It has been shown that the preparative ultracentrifuge can be used to remove virus from dilute suspensions with relatively high quantitative efficiency (4, 5). The disadvantages of this method are the high cost per sample and the limited sample capacity of the equipment. The method to be described in the present study combines both technics in an attempt to take advantage of their best features while limiting their disadvantages. The nominal concentration factors achieved by the method may be computed in two ways, both of which are based upon the reduction of sample quantity. In the conservative approach, one considers that a standard 25-gm. sample of the food itself is being reduced to a volume of 0.5 ml. preparatory to testing and, therefore, that the volume reduction has been 50-fold. Since the volume of clarified food extract obtained from the 25-gm. sample exceeds 100 ml., one might also conclude that when this volume had been reduced to 0.5 ml., a concentration of 200-fold had been achieved. These are called nominal concentration factors because they disregard any loss of virus which may result during the concentration process. Thus, a 200-fold reduction in sample volume with a 50% virus loss, would result in a true net increase of only 100-fold in the titer of virus per unit sample volume. In a system in which the original fluid food slurry might be tested directly in tissue cultures if enough of the latter were available, it is the net concentration factor achieved (based on virus loss and volume reduction) which determines the advantage gained by concentration. A further limitation upon the concentration of food samples for virus detection is that both the polyethylene glycol and ultracentrifuge methods are subject to physical interference by food components.

It is therefore necessary that the original food suspension or slurry be clarified to remove these food components before beginning the concentration process.

The clarification step in processing food samples may present greater problems than the concentration itself. Ideally, one is attempting to remove virtually all of the food solids from the fluid suspension while losing none of the virus. Many of the clarification methods tested in the course of the present study have fallen considerably short of this ideal. First, it has been found that virus in the suspension may be removed together with the food solids and discarded. Second, some of the treatments employed in clarification may inactivate the virus, so that although it is still physically present in the final preparation, it is no longer capable of producing a demonstrable infection. Finally, it has been noted that the costs involved in clarification (particularly, labor costs) may be greater than those involved in the subsequent concentration.

V. MATERIALS AND METHODS

The preparation of Coxsackie virus, type A9, strain P.B. (Bozek), employed in these studies has been described previously (4). The plaque titration method which had been used in our laboratory (2, 4) was modified to include two washings of the primary rhesus monkey *(Macaca mulatta)* kidney cell monolayer with 5 ml. of phosphate-buffered saline (PBS) at the end of the adsorption period. This was necessitated by the presence of food residues in some of the samples which, if not removed, would be trapped in the agar and would tend to obscure the plaques.

A general approach was common to all of the procedures tested. A known quantity of virus was added to 25 gm. of creamed cottage cheese which had been bought at retail. This model *sample* was homogenized in aqueous diluent plus other additives and centrifuged to remove as much as possible of the food solids. The supernatant fluid *(food extract)* was concentrated by a sequential process of dialysis against polyethylene glycol followed by ultracentrifugation. The pellet in the ultracentrifuge tube was collected in about 0.4 ml. of PBS diluent and tested in a single tissue culture by the plaque technic.

Criteria for adequacy of clarification of the food extract were subjective. It was concluded that clarity was sufficient if the extract was opalescent rather than opaque or translucent, if the pellet resulting from ultracentrifugation was barely visible, and if the concentrated sample did not intoxicate the cells of the culture in which it was tested. These criteria were applied sequentially to clarification methods under study, and each technic was abandoned or modified if found unsatisfactory. Toxicity problems were never entirely eliminated. Finally, clarification methods which appeared adequate were tested with slurries of virus-contaminated cottage cheese. The goal in this case was the highest possible volume yield of clear extract with the highest possible virus titer. Only limited attempts were made to determine whether virus loss was due to removal with the food solids or to inactivation.

The concentration process combining dialysis against polyethylene glycol with ultracentrifugation was adapted from technics described previously. Polyethylene glycol (Carbowax 20000, Union Carbide) was melted in an autoclave (121° C., 30 min.) and cast into cylinders 1.5 cm. x 100 cm. by pouring it through a polypropylene powder funnel to which a length of dialysis tubing had been fitted. When hard, the casting was stripped and cut into 16-cm. lengths (approximately 30 gm.). One of these *slugs* was inserted into an 80-cm. length of dialysis tubing which was doubled back upon itself. A special funnel consisting of the conical portion of a 500-ml. Erlenmeyer flask fused to a 25-mm. (o.d.) cylindrical neck was inserted into the mouth of a polyallomer tube for the No. 30 rotor of the Spinco Model L ultracentrifuge, and the dialysis tube containing the *slug* was passed through the neck of the funnel and down into the ultracentrifuge tube. The fluid extract of a 25-gm. cottage cheese sample (about 105 ml.) was poured into the funnel. After 10 to 16 hours at 35° to 37° C., the volume of the extract had been reduced to equal to or less than 25 ml. by imbibition of water and solute into the dialysis tube. The outer surface of

the dialysis tube and the inner surface of the funnel were rinsed with a small volume of deionized water, which ran down into the concentrated sample. The dialysis tube and funnel were removed; and the ultracentrifuge tube was capped, filled the rest of the way with PBS, and run for 5 hours at 105,000 × g.

Three detection methods reached the point in development at which it was thought worthwhile to determine their end point sensitivities. Samples were inoculated with 2-fold dilutions of virus at levels of less than 1 plaque-forming unit (PFU) per gram of the food. Comparable inocula were held in the refrigerator while the detection process was carried out, and the control virus suspensions and the concentrated samples were tested simultaneously in tissue cultures. Results were expressed as 50% end points, as has been done previously (2, 3, 4). The details of each of these three methods have been tabulated. In method A, details of which are given in table I, the most significant feature was the use of 100 ml. of Freon TF in step 1. The remainder of the procedure was predicated upon the use of this additive. Addition of bentonite to the mixture in step 1, as in method B, required a change of diluent composition but permitted simplification of the concentration process, as is shown in table II. Method C, detailed in table III, included an additional treatment of the food extract with another 100 ml. of Freon TF.

VI. RESULTS

Clarification

It was determined early that a fair degree of clarity could be achieved if the slurry, consisting of 25 gm. of cottage cheese in 100 ml. of PBS, homogenized for 2 minutes at top speed in a Servall Omnimixer immersed in an ice bath, was centrifuged for 30 minutes at 32,000 × g. Losses of virus titer resulting from this treatment were not statistically significant ($P > .05$) with the present model agent, but it had been found previously that losses in excess of 90% would have been experienced if a larger virus had been employed. Means were therefore sought by which equal or better clarity could be obtained without resort to such extreme gravitational force.

Several approaches were tested and discarded. These will be reviewed, briefly, to save others the trouble of repeating them. Both depth filters and surface-retention (membrane)

TABLE I

Outline of detection method A

Step	Substance	Treatment
1	Contaminated cottage cheese (25 gm.) Phosphate-buffered saline (pH 7.4) (100 ml.) Freon TF (100 ml.)	Homogenize in 400 ml. Omnimixer cup in ice bath, 2 min. at top speed.
2	Homogenate	Centrifuge for 30 min. at 5,600 × g, discard sediment.
3	Supernate	Concentrate 10 hr. or more against 30 gm. polyethylene glycol at 37° C., rinse surfaces of bottle and dialysis tubing.
4	First concentrate	Centrifuge 5 hr. at 105,000 × g, discard supernate.
5	Pellet	Resuspend in 7 ml. PBS with 5 glass beads on a Vortex mixer.
6	Second concentrate	Centrifuge 5 min. at 1,500 × g, discard sediment.
7	Supernate	Centrifuge 2 hr. at 198,000 × g, discard supernate.
8	Pellet	Resuspend in 0.5 ml. PBS + 2% agamma chicken serum.
9	Third concentrate	Test in tissue culture.

TABLE II

Outline of detection method B

Step	Substance	Treatment
1	Contaminated cottage cheese (25 gm.) Glycine-NaOH buffer (pH 8.8) (100 ml.) $MgCl_2 \cdot 6\,H_2O$ (20 gm.) Bentonite (10 gm.) Freon TF (100 ml.)	Homogenize in Servall 530 centrifuge cup on Omnimixer, 2 min. at top speed in ice bath.
2	Homogenate	Centrifuge 30 min. at 8,000 × g, discard sediment.
3	Supernate	Concentrate 10 hr. or more against 30 gm. polyethylene glycol at 37° C., rinse surfaces of funnel and dialysis tubing.
4	First concentrate	Centrifuge 5 hr. at 105,000 × g, discard supernate.
5	Pellet	Resuspend in 0.5 ml. PBS + 2% agamma chicken serum.
6	Second concentrate	Test in tissue culture.

TABLE III

Outline of detection method C

Step	Substance	Treatment
1	Contaminated cottage cheese (25 gm.) Glycine-NaOH buffer (pH 8.8) (100 ml.) $MgCl_2 \cdot 6\,H_2O$ (20 gm.) Bentonite (10 gm.) Freon TF (100 ml.)	Homogenize in Servall 530 centrifuge cup on Omnimixer, 2 min. at top speed in ice bath.
2	Homogenate	Centrifuge 30 min. at 8,000 × g, discard sediment.
3	First supernate	Homogenize with 100 ml. Freon TF as in step 1.
4	Homogenate	Centrifuge 15 min. at 8,000 × g.
5	Second supernate	Concentrate 10 hr. or more against 30 gm. polyethylene, glycol at 37° C., rinse surfaces of funnel and dialysis tubing.
6	First concentrate	Centrifuge 5 hr. at 105,000 × g, discard supernate.
7	Pellet	Resuspend in 0.5 ml. PBS + 2% agamma chicken serum.
8	Second concentrate	Test in tissue culture.

filters were tried. These were first tested with supernatant fluids obtained by centrifuging slurries at 2,000 × g for 30 minutes. No grade, or combination of grades, of paper filters was found through which these fluids would pass without rapid plugging. The same was true when membrane filters or Seitz EK pads were employed; nor did Hyflo Super-Cel (Fisher Scientific), a general-purpose filtering aid, assist significantly. Unless the fluid to be filtered had already been clarified sufficiently to pass freely through the filters, plugging and the resulting reduction of functional pore size caused virus losses exceeding 90%. Additives to the homogenization mixture, intended to facilitate removal of the food solids at moderate

centrifugal forces, were also tested. Those which proved useful will be described below. Additives which were rejected, either because they did not facilitate clarification or because they did so no better than others already in use, included: n-butanol; chloroform and bentonite, alone or in combination; diethyl ether with or without bentonite, Freon TF, or both; Freon TF alone followed by bentonite alone in a second extract; and bentonite alone followed by Freon TF with or without bentonite in a second extraction.

Addition of 100 ml. of Freon TF (1,1,2-trichloro-1,2,2-trifluoroethane, DuPont) to the mixture to be homogenized was among the earliest approaches. A variety of aqueous diluents was tested in homogenization mixtures employing Freon TF; and it was found that deionized water, phosphate buffer, or PBS would serve equally well. When cottage cheese was contaminated with sufficient virus to permit quantitative comparisons, the titer of the supernatant fluid obtained after steps 1 and 2 of method A did not differ significantly from that of aqueous food slurry. The same had been true when an aqueous food slurry prepared in the absence of Freon TF was centrifuged at 32,000 × g for 30 minutes; however, the supernatant volumes (and, therefore, the net virus recoveries) obtained by these two methods were 80% and 50%, respectively, of the total initial volume of diluent plus food. This represented a considerable gain in quantitative efficiency, with a significant moderation in the required centrifugal force, but it was found that a sufficient quantity of food solids remained in the food extract to prevent concentration to a final volume of 0.5 ml. unless additional treatment as in steps 6 through 9 of method A was employed. This additional process is sometimes called *differential centrifugation*, and it removed the residual food solids quite effectively. Because the pellet (step 5) could not be completely dispersed, even by sonic treatment, it also removed some of the virus. Therefore, still more efficient clarification methods were sought.

When the combination of Freon TF and bentonite shown in step 1 of method B was first tested, more than 90% virus loss resulted. This was true both when M/15 phosphate buffer and when PBS was used as the aqueous diluent in homogenization. Trial and error led to a diluent consisting of 100 ml. of a 0.09 *M* glycine + 0.01 *M* NaOH solution to which were added 20 gm. of $MgCl_2 \cdot 6H_2O$. The pH of this diluent was approximately 8.8. The volume of the supernatant fluid obtained after step 2 of method B was consistently 80% of the total volume of food plus diluent, and the titer was usually slightly higher. If M/15 phosphate buffer at pH 7.7 was substituted as the diluent, or if the quantity of $MgCl_2 \cdot 6H_2O$ was reduced to 10 gm., or both, the titers of the resulting food extracts were found to be 36%, 76%, and 64%, respectively, of that obtained with the formulation given for method B. The end-point sensitivity of method B was encouraging, though the quantitative efficiency with which virus was recovered was low. A number of the cultures inoculated with the concentrated samples were found to be intoxicated, so one further modification was tested.

It can be seen, by comparison of tables II and III, that the procedural difference in method C is the addition of a Freon TF treatment of the first food extract. This was found to produce a greater degree of clarity, judged either by the appearance of the food extract or by the size of the pellet following ultracentrifugation, with little or no virus loss in this step and possibly a slight gain in the quantitative efficiency of virus recovery for the entire detection method. When bentonite, as well as Freon TF, was added in step 3, greater than 90% virus loss resulted.

Concentration

The advantages of reversing the polyethylene glycol concentration so that the sample was outside, rather than inside, the dialysis tubing were several. First, the ease of handling the polyethylene glycol was greater both at the start and at the end of the process. Second, residues of sample could be rinsed from the outside of the dialysis tubing without requiring the special equipment which had had to be used in recovering samples from inside the tubing (2). Third, the extent of concentration could be controlled by the depth to which

the loop of dialysis tubing extended into the sample. Finally, the special funnel assembly made it possible to carry out the final, ultracentrifuge concentration without transferring the sample from one vessel to another. The principal disadvantage, when compared to the previous approach, was that longer times and higher temperatures (due to diminishing contact surface areas) were required. The presence of approximately molar magnesium chloride in the slurry appeared to protect the model virus from inactivation during concentration, but it remains to be seen whether viruses of other groups will do as well.

Because the ultracentrifuge tube forms part of the original container in which polyethylene glycol concentration was performed, it was not possible to employ the gelatin *traps* which had been used previously in ultracentrifugation of viruses from laboratory diluents (4, 5). The intended purpose of these traps was principally to prevent virus loss due to inertial restirring during deceleration of the ultracentrifuge rotor. The pellet of residual food solids formed in the present system seemed to serve the same purpose, though it was somewhat more difficult to resuspend than the gelatin, particularly in the case of method A.

Detection

The plaque technic was employed to detect virus in these experiments because it provided both qualitative and quantitative information. The practice of washing the cell sheet at the end of the adsorption period served to remove any gross food solids which might have been present in the inoculum and also to reduce the microbial population in the culture. The market cottage cheese to which the virus was added was not sterile, nor were aseptic procedures used during the processing of the samples. With reasonable care to minimize contamination in handling, it was found that washing the cells, plus the normal level of antibiotics in the overlay medium, sufficed to prevent visible contamination of the cultures. The intoxication of occasional cultures which has been observed has never been satisfactorily explained, but its incidence was apparently lower with samples prepared by method C than by method B, and lower still when concentrated food solids were removed before testing, as in step 6 of method A.

Sensitivity

It has been stated above that approximately a 20% virus loss could be anticipated in the clarification step of each of these methods. Demonstration of virus losses in other steps has been extremely difficult when these have been tested individually at levels of contamination exceeding 10 PFU/gm. of sample. Nevertheless, it was reported previously that the quantitative efficiency with which virus was recovered at lower contamination levels, even in the absence of food solids, was significantly decreased (2, 4, 5). Low-level contamination will be defined in the present case as less than 1 PFU/gm. of sample. It will be shown that the quantitative efficiency with which virus is recovered in this range is relatively low and that the quantity of virus which must be present in a sample to produce a 50% probability of detection is thereby increased.

Results of two small experiments in which method A was tested have been combined and summarized in table IV. The data presented are too limited to allow precise computation of the parameters associated with method A, but it should be noted that the 50% end point was approximately 10 PFU/sample and that about 7% of the virus put into the food was recovered. None of the cultures used to test the concentrated food extracts was intoxicated and killed.

Intoxication and killing of cultures occurred with significant frequency in tests of method B. Three sets of 12 samples were processed, with the results shown in table V. Two interpretations of these data are possible, depending upon whether one chooses to include the intoxicated, dead cultures in the calculations. The rationale for excluding the dead cultures from consideration is that, had a test procedure other than the plaque technic been employed, these cultures might have been spared, and some of them might have yielded positive results. If the tests in which the cultures died are considered to be negative, the 50% sensitivity end point of method B is approximately

TABLE IV

Detection of low-level virus contamination by method A

Contamination range (PFU)	Samples tested	Qualitative findings		Quantitative findings	
		Positive	Negative	Controls	Recovered
				(PFU)	
8-16*	6	4	2	59	6
4-8	2	0	2	15	0
2-4	2	0	2	8	0
All	10	4	6	82	6

*Based upon mean control count ($\bar{X}$): $8 < \bar{X} < 16$.

TABLE V

Detection of low-level virus contamination by method B

Contamination range (PFU)	Samples tested	Qualitative findings			Quantitative findings		
		Positive	Negative	Dead	Controls	Adjusted	Recovered
					(PFU)		
> 16	3	3	0	0	80	80	24
8 - 16	3	1	1	1	37	25	3
4 - 8	9	7	1	1	55	47	14
2 - 4	9	2	5	2	32	27	2
1 - 2	6	0	6	0	12	12	0
< 1	6	1	4	1	4	3	1
All	36	14	17	5	220	194	44

4.5 PFU sample, and about 20% of the virus was recovered. The 50% end point may be estimated at 3.9 PFU/sample with the dead cultures excluded, but the computation of quantitative efficiency becomes somewhat more complicated. In table V, a column has been added which gives the number of plaques seen in the cultures receiving the control inocula, adjusted to the number of PFU probably present in the food samples whose concentrated extracts did not kill the test cultures. The adjusted quantitative efficiency was found to be approximately 22%.

Two sets of 12 samples were processed and tested by method C, with the results shown in table VI. It can be seen that only 1 of 24 test cultures was killed and that the influence upon the interpretation of the results is, therefore, relative slight. The 50% end point for method C is approximately 3.9 PFU/sample, and the quantitative efficiency is about 35%, with all samples included. Excluding the only sample which killed its test culture, the corresponding values are approximately 3.7 PFU/sample and 36% recovery.

Two further experiments were performed in an effort to define more adequately the difference in the efficiencies of methods B and C. In each, 12 samples were contaminated at a single level, and 6 were processed by each

TABLE VI

Detection of low-level virus contamination by method C

Contamination range (PFU)	Samples tested	Qualitative findings			Quantitative findings		
		Positive	Negative	Dead	Controls	Adjusted	Recovered
						(PFU)	
8 - 16	3	3	0	0	26	26	9
4 - 8	6	4	2	0	32	32	15
2 - 4	6	2	3	1	15	13	2
1 - 2	3	1	2	0	3	3	1
≤ 1	6	0	6	0	1	1	0
All	24	10	13	1	77	75	27

TABLE VII

Comparison of methods B and C at low contamination levels

Test method	Samples tested	Qualitative findings			Quantitative findings		
		Positive	Negative	Dead	Controls	Adjusted	Recovered
						(PFU)	
B	12	5	2	5	51	33	15
C	12	10	1	1	51	50	23

method. The levels of contamination were found to have been approximately 1 PFU/ sample and 7.5 PFU sample, respectively. The combined results are presented in table VII. With all cultures included, the quantitative recovery by method B was estimated at 29% while that for method C was 45%. The adjusted estimates are 45% and 46%, respectively. Clearly, the results of these additional experiments suggest that the adjusted quantitative efficiencies of virus recovery for the two methods may not differ to nearly the extent indicated by the data of tables V and VI. The only contrast which is considerably reinforced by these additional data is that concerning the frequency with which cultures are intoxicated. The validity of adjusting the efficiency estimates depends entirely upon whether methods of testing in tissue culture, other than by the plaque technic, really avoid the toxicity problem and whether the results which may be obtained by other test technics are at least as useful as plaque counts in the application of the method which is contemplated.

VII. DISCUSSION

The 25-gm. samples of cottage cheese employed in these studies could be expected to contain approximately 6 gm. of food solids, of which most is protein (6). The methods developed have been intended to separate from this, as completely as possible, less-than-microgram quantities of virus. This goal has been at least partially achieved. The bulk of food solids present in the original sample is so great that concentration to less than 0.5 ml. would be impossible unless the greater part had been removed. Still, enough of the food remains in the clarified extract to interfere with the adsorption of virus to filter membranes; and this, in turn, has ruled out the use of adsorption to membranes and elution, as had been

done previously with water samples (3), as a means of concentrating the virus.

The data presented indicate that a reasonable degree of sensitivity has been achieved. The 50% sensitivity end point for both method B and method C is less than 5 PFU/sample, or less than 0.2 PFU/gm. of food. As was stated in the introduction, the utility of achieving this degree of sensitivity by this means must ultimately be judged by whether cost savings result. The standard of comparison is the simplest possible detection method, which eschews concentration by testing the entire volume of food extract, after minimal clarification, which is required to achieve a comparable level of sensitivity. As little as 20 ml. of extract from cottage cheese contaminated at 0.2 PFU/gm. could be expected to provide a level of sensitivity comparable to method B, and this would require approximately 40 flask tissue cultures if it were to be tested without concentration.

Analyses of direct costs indicate that flask cultures produced by our methods are worth about $1 each, so the total cost of testing such a sample without concentration would exceed $40. It has been found that methods B and C may be carried out on a routine basis and will accommodate 12 samples per day. Assuming routine testing of 12 samples per day, with full utilization of labor at other tasks when not actually engaged in the performance of these procedures, the total costs of labor, materials, and depreciation of equipment have been estimated at $3.88 per sample for method B and $4.43 per sample for method C. The costs of everything but the tissue culture might be increased by as much as 100% in a less efficient operation, in which case the totals would be $6.76 and $7.86 per sample, respectively. It thus appears that concentration by this method makes possible a significant saving in the cost of testing a sample. It should also be noted that 480 flask cultures per day would be consumed in testing 12 samples. These cultures are observed daily from the second through the sixth or seventh day after inoculation, so that 2,400 cultures would have to be observed each day to meet the same schedule.

The work reported here has been done with a single model system. If the methods described are to be employed in the testing of field specimens, much more must be learned concerning their suitability for other foods and other virus contaminants. Studies intended to produce the necessary information are already under way. This is one reason why judgment has been withheld as to whether the small gain in efficiency of method C compared to method B warrants the additional trouble and cost. Preliminary data indicate that foods may be found for which the additional Freon TF treatment is essential and others for which it is of no value whatever.

It is possible that these methods could also be applied to the detection of viruses in clinical specimens, such as feces or blood clots, and in infected tissues. Perhaps only in special circumstances would the levels of sensitivity attainable by these methods be required in clinical applications. Compromise methods, by which lesser sensitivities might be achieved at a lower cost per sample, may also merit study.

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APPENDIX I

TISSUE CULTURES

The tissue cultures employed in the present detection technic are the key to the entire system, for the most elegant detection procedures will ultimately be thwarted if the prepared sample is inoculated into an inappropriate or inadequate tissue culture. On the other hand, it should be realized that a single tissue culture provides no more information to the virologist than a single tube or plate of medium does to the bacteriologist. With this perspective, it is easy to see that the tissue culture is only a tool and that its costs should, therefore, be minimized to the greatest extent possible.

Choice of culture. Primary monkey kidney cell cultures, such as have been used in the present study, are widely employed in the detection of viruses from clinical specimens. Both the cultures and the animals from which they may be produced are generally available, and the cultures themselves have a relatively broad spectrum of susceptibility to viruses infectious for humans. The principal liability of these cultures is that they are sometimes found to contain adventitious viruses of simian origin. Some such viruses seem to have little effect upon the utility of the cultures for detection of viruses, while other adventitious agents may render the cultures totally useless or may make it impossible to grow cultures from the kidneys of certain monkeys. The cost of testing procedures required to rule out completely the presence of simian agents from these cultures is prohibitive. Therefore, when simian viruses have been shown or presumed to be causing problems in our tissue culture operation, we have simply changed monkey sources temporarily. This has resulted in some delays in scheduling the laboratory work, but has never failed to circumvent the problem.

Method of collection. Kidneys for culture are collected from adolescent or young adult rhesus monkeys *(Macaca mulatta)*, preferably weighing at least 5 pounds. The animal is anesthetized by intraperitoneal injection of approximately 6 to 8 ml. of a 60% solution of chloral hydrate, and is laid supine. A long midline incision is made in the abdomen, and the viscera are displaced from the abdominal cavity. The renal artery and vein and urethra are clamped collectively on each side with a 6-inch, curved Kelly hemostat. All three vessels are cut near the hilus of the kidney (distal to the clamp), and the kidney is removed from the retroperitoneal cavity with the capsule intact. The viscera are replaced in the abdominal cavity, and the hemostats are removed. In this way the animal dies promptly of arterial bleeding without regaining consciousness and without bleeding on the bench-top. This portion of the procedure is best carried out somewhere other than in the tissue culture laboratory. The kidneys, still in their capsules, are immersed in a buffered hypochlorite solution in a beaker and carried to the tissue culture laboratory. This effectively disinfects the surface of the capsule and eliminates the requirement for aseptic procedure in collecting the kidneys from the animal. At the tissue culture laboratory, the capsule surface is rinsed with saline *Y* (4), and the capsule is removed aseptically from the kidney. Each kidney is cut into four pieces, and all of the pieces are placed in a 50-ml. short-taper centrifuge tube. These are minced with long-handled, sharp-pointed surgical scissors to pieces whose greatest dimensions are 2 to 4 mm. The tissue pieces are rinsed three times with saline *Y* before trypsinization.

Trypsinization. The apparatus we use in our laboratory permits trypsinization to be carried out as a flow process on the laboratory bench-top with reasonable control of temperature. The method has been found to enhance cell yields by as much as 50% over a comparable batch trypsinization process, other things being equal. It also cuts labor costs by permitting the tissue culture technician to do other things while the trypsinization is in process. Trypsin is purchased as a sterile, lyophilized preparation (Baltimore Biological Laboratories) and is reconstituted and diluted to a final concentration of 0.25% in saline *Y*. Saline *Y* was especially developed for this application: it is a salt solution free of divalent cations, which is strongly buffered

with phosphate, but which allows for some pH adjustment with bicarbonate buffer just before use to permit greater flexibility in application. A total of 2,400 ml. of trypsin solution containing antibiotics in standard concentrations (4) is prepared in a 3-liter flask. The trypsin solution passes from the flask through a heat-resistant Tygon tube ($^3/_{16}$-inch, i.d.), the end of which is weighted to the bottom of the flask with a stainless steel sinker. Flow of trypsin through the apparatus is induced and controlled by threading the tubing through a Sigmamotor (model TT 8) peristaltic pump which is driven with a Zero-Max (model E1) continuous speed control unit. The trypsin then flows up through a 200 mm. Graham-style condenser and down into the Rappaport continuous-flow trypsinizing flask (Bellco Glass Inc.). The condenser is mounted vertically, parallel to the trypsinizing flask, and through its jacket is passed water at 37° C. from a constant temperature circulator. This allows the reservoir of trypsin solution to be kept cold, so as to minimize loss of activity during the course of the trypsinization, and yet provides accurate control of the temperature of the trypsin solution as it enters the active portion of the apparatus. Because the trypsinizing flask is not insulated, it is assumed that the temperature at which the process is carried out is somewhat below 37° C., but the design of the flask is such as not to permit measurement of the internal temperature. In the trypsinizing flask have been placed the pieces of kidney tissue, minced and washed as described above, and 100 ml. of trypsin solution. This is agitated for 15 minutes at the greatest possible speed by means of a magnetic drive unit and a 1-inch Teflon-coated magnet bar, and the first trypsin suspension is discarded. The flow rate through the apparatus is subsequently adjusted to approximately 15 ml./min. The effluent cell suspension in trypsin passes through the bottom of the flask and through a length of heat-resistant Tygon tubing ($^3/_8$-inch, i.d.), into a new 32-oz. prescription bottle in an ice bath. The top of the prescription bottle is shielded from contamination by threading the Tygon tubing through the stem of a glass powder funnel which is inverted over the top of the bottle. Fifty ml. of calf serum are placed in the receiving vessel before trypsinization is begun. After approximately 30 minutes, the volume of cell suspension plus serum has reached the 800-ml. mark on the bottle. This bottle is replaced with another which also contains 50 ml. of calf serum. The collected cell suspension is distributed among 4 centrifuge bottles (200-ml., conical-bottom, screwcap) (Bellco Glass Inc.) and centrifuged for 13 minutes at 900 r.p.m. and a temperature of 5° C. in an International PR 2 centrifuge. The sedimented cells are resuspended in a small volume of saline *Y* and sedimented again. These washed cells are suspended in 800 ml. of growth medium consisting of Hanks's balanced salt solution, 0.5% lactalbumin hydrolysate, and 5% calf serum with the standard concentration of antibiotics. Cells from subsequent harvests are collected, washed, and suspended in this same bottle of growth medium until all of the prepared trypsin has been expended, the cell suspension in growth medium being held in the refrigerator until the process has been completed. The suspension is then counted with a hemacytometer and diluted with additional growth medium to a final concentration of 300,000 cells per milliliter. The final cell suspension is placed in an Erlenmeyer flask and kept under continuous magnetic agitation while the cells are dispensed into culture vessels. Dispensing is done with a siphon such as that described for the trypsin reservoir, attached with stainless steel Luer adapters to a Filamatic DAB pipetting machine. Five ml. of cell suspension are dispensed into each flask (Falcon plastics, No. 3012). These flasks are laid singly on the shelves of a vibration-free 36° C. incubator for at least 48 hours to permit uniform attachment of the cells. Thereafter, the flasks are stored and handled in stacks of 4, held together by a rubber band, until the time of virus inoculation.

Media preparation. With one exception, media are made up with deionized water from dry powder preparations purchased from the Grand Island Biological Company. The exception is that Eagle's minimal essential medium (MEM) is prepared by reconstituting from dry powder Earle's balanced salt solution, and sterilizing it in the autoclave. To this are added sterile, commercially prepared concentrates of the amino acids and vitamins required to complete the medium. Heat-labile media such as 199 and L-15 are sterilized by filtration through 0.22 μ porosity Millipore or 0.20 μ porosity Gelman membranes in a 142 mm. Millipore stainless steel filter holder. Filtration is carried out under line air pressure of 25 to 30 p.s.i. with the medium in a reservoir consisting of a double sidearm vacuum flask (4 liters) (Bellco Glass

Inc.). Medium passes into this flask through a 1-liter separatory funnel fitted to a bored stopper at the top. This stopper is held in place by a *hold-down* improvised of a worm gear hose clamp and heavy gage wire. When the medium has passed into the filtration flask the stopcock of the separatory funnel is closed and air pressure is introduced through the upper sidearm of the flask. The medium flows out through the lower sidearm and through tubing to the filter holder. The effluent passes through rubber tubing to a glass tip, shielded by another glass powder funnel, and is dispensed directly into 16-oz. prescription bottles for storage. Media sterilized by autoclaving are stored at room temperature until use, while media which must be filter-sterilized are stored at 5° C.

Medium change procedure. Two or three days after seeding, the medium in the cultures is changed. Medium of the same formulation is used except that the quantity of bicarbonate is increased slightly. Medium is dispensed from the prescription bottle in which it was stored, using a specially made siphon. To one hole of a 2-hole rubber stopper is fitted a "breather" consisting of a glass wool–packed bulb blown in polypropylene tubing. Through the other hole of the stopper passes a glass or polypropylene tube extending from the bottom of the bottle to a length of rubber tubing on the outside which ends in a stainless steel Luer adapter. This is connected to a 5-ml. syringe on the left side of the Filamatic DAB apparatus. Medium passes from the syringe to a double 14-gage needle fabricated especially for us by Becton-Dickinson. The second of the paired needles is connected to heat-resistant Tygon tubing with another Luer adapter. This tubing attaches to the right-hand side of the Filamatic apparatus which has a 20-ml. syringe set for approximately a 10-ml throw. Effluent from this syringe is led through tubing to a sinker which may be placed in a receiving flask or beaker, or simply in the sink drain. The Filamatic machine is equipped with a single-cycle attachment and a foot-switch. It has been found more efficient to operate the foot-switch with the heel of the hand than with the foot. The medium change procedure consists of removing the screwcap from the flask (which is held cell-side-up) and inserting the rigidly mounted double needle to the lowest corner of the flask. The foot-switch is actuated, and the pipetting machine makes one complete revolution and stops. During the first half of the revolution both syringes fill with medium, that on the left with fresh medium and that on the right with the spent medium which is aspirated from the flask through one of the paired needles. During the second half of the revolution, both syringes empty; the fresh medium on the left side syringe is emptied into the flask, while the spent medium is emptied through the exhaust tube into the receiving vessel or the sink drain. By handling the flasks in stacks of 4 held together with a rubber band, as was described previously, an operator can change 200 or more flask cultures per hour with a little practice. Further medium changes are performed usually on days 5 and 7. Medium used on day 5 consists of 49% Earle's balanced salt solution with lactalbumin hydrolysate, 49% medium 199, and 2% agamma calf serum (Hyland Laboratories). By the seventh day the cultures are usually confluent and ready for use. If they are to be used quite promptly, they are changed to L-15 medium with 2% agamma calf serum and a small amount of bicarbonate, and are incubated at 37° C. Cultures which are likely to be held for longer periods of time (up to 3 weeks) before use, are maintained at room temperature. In this case, the preferred maintenance medium consists of 49% medium 199, 49% MEM, and 2% calf serum. The latter medium is prepared with only three-fourths of the formula concentration of sodium bicarbonate. Since the cells do not metabolize actively at room temperature, this maintains the pH and compensates for the tendency of carbon dioxide to escape through the plastic of the flask during prolonged storage. Agamma calf serum is used exclusively in media during the late stages of culture growth. This serum has been found to be devoid of inhibitors for enteroviruses, and by using it in the last one or two medium changes, it effectively washes away any residual inhibitors which might be present from the normal serum used in the previous media. Although data would be extremely hard to obtain, it has seemed to us that the normal calf serum is slightly superior for starting new primary cultures.

Plaque technic. Our method for assaying viruses by the plaque technic has been described previously (2, 4). In brief, the maintenance medium is emptied from the

flask cultures and each is inoculated with approximately 0.5 ml. of virus suspension. Where serial dilutions of a virus sample are to be tested, the dilutions are carried out in 1.8-ml. dilution blanks consisting of phosphate buffered saline with 2% agamma chicken serum (Hyland Laboratories). These are prepared in disposable 13 x 100 mm. tubes with disposable plastic caps. The quantity of inoculum for a 10-fold dilution is, obviously, 0.2 ml. The contents of the tube are mixed on a Vortex mixer, and 1.2 ml. are withdrawn with a cotton-plugged bacteriologic pipet which is graduated at 0.5, 1.0, 1.1, and 1.2 ml. This permits dispensing 0.2 ml. into the next dilution blank and 0.5 ml. into each of 2 flasks at each dilution level. The use of these pipets has been found to expedite considerably the performance of these serial 10-fold dilutions. The inoculated cultures are held for 2 hours at room temperature in continuous agitation on a Bellco rocker platform. It has been found that room temperature adsorption is optimal for the enteroviruses. With adsorption at 37° C., highest plaque counts are obtained after a 1-hour period; and, thereafter, longer adsorption seems to result in lower titers. The exact length of the adsorption period is much less critical when done at room temperature, but the plaque count for any period from 90 to 180 minutes of adsorption exceeds that obtained after 60 minutes of adsorption at 37° C. At the end of the adsorption period, the cells are washed if there is anything in the inoculum which may interfere with subsequent counting of the plaques. The washing fluid consists of 5 ml. per flask of phosphate buffered saline with 2% agamma chicken serum. Overlay is added with a siphon bottle like that described above, and a 5-ml. Cornwall syringe. The overlay medium is compounded in somewhat unusual fashion. A 2-fold concentrate of the basic medium is mixed of 3 solutions, in the indicated proportions, which have been autoclaved separately. The first consists of 300 ml. of deionized water in which are suspended, as completely as possible, 0.4 gm. of protamine sulfate. The second is 100 ml. of a 10-fold concentrate of Earle's balanced salt solution. The third is 100 ml. of a 10 m*M* cysteine + 0.25 *M* $MgCl_2$ solution in deionized water. Shortly before dispensing, the medium is mixed in final proportions and held in a 45° C. water bath. The final proportions consist of 50 ml. of the 2-fold medium concentrate; 33 ml. of a 3% solution of Noble agar (Difco); 2 ml. of agamma calf serum; stock solutions of neutral red, antibiotics, and sodium bicarbonate; and enough sterile, deionized water to make a final volume of 100 ml. Noble agar for this purpose is dispensed in dry form into milk dilution bottles calibrated at 99 ml., using a Lyman Ideal gunpowder measure set to drop 3 gm. of agar. With practice, an operator can dispense agar within ± 0.1 gm., and the method is much more rapid than weighing. When the powder measure has been set up, dry agar is rapidly dispensed into 20 to 30 clean bottles, which are capped and stored at room temperature until needed. When agar is required, deionized water is added to the bottle to the graduation mark on the side, and the entire bottle is placed in the autoclave at 121° C. for 15 minutes. The result is a dissolved, sterile, 3-fold concentrate of agar, ready to use in mixing the final overlay medium.

Once the overlay has solidified in the cultures, the flasks are inverted and incubated at 36° C. and observed for plaques daily from the second through the seventh days. Plaques are marked permanently as soon as they are observed, with a fine-point, wick-tip pen. A different color of mark is used for each day's observations to ensure that each mark truly designates a plaque. Thus one can distinguish during observations on the sixth day between plaques which were first observed on the day previous, and those which were first observed 3 days before. If the plaque designated by a 3-day-old mark is still at the limit of visibility, it is concluded that it was not really a virus-induced plaque (which would have continued to increase in diameter during subsequent incubation), but rather an imperfection in the cell sheet. After all previously marked plaques have been inspected to insure that they are legitimate, the plaques which are new that day are marked and counted, and their number is added to the previous total and recorded. This system has been found to increase the speed and accuracy of observations.

Tissue cultures can constitute a major cost in the virus laboratory; the system which has been described was developed to minimize the cost of these cultures. For those not wishing to produce their own cultures, comparable ones can be obtained commercially for $2.75 or more. Where tube, rather than flask cultures are to be

employed, commercial prices are considerably lower. The volume of inoculum which can be tested in a single tube culture, however, is approximately one-fifth of that which can be tested in a flask culture. Tubes cannot be used for plaque titrations nor with phase contrast microscopes, except with special equipment to compensate for the curvature of the tube. Even so, permissible magnifications are extremely limited. Obviously, not all of the unusual technics described in this section were originated in our laboratory. They have been described here in detail to provide a basis for the cost figures presented later in the report and in the hope that at least some of these innovations might prove useful in other situations.

APPENDIX II

COST ESTIMATES

It was stated in the introduction that the use of concentration in any detection method for food-borne virus, with its attendant requirement for extreme clarity of the sample extract, could be justified only if the costs involved were less than those which would be incurred if the sample slurry were tested without concentration (in the requisite number of tissue cultures) to produce a similar level of sensitivity. A number of arbitrary assumptions must be made in order to permit such a comparison. Some of the arbitrary limits are imposed by the equipment employed in what has now been designated the standard testing method. These include the stipulation of a 25-gm. sample size and a capacity of 12 samples per day, based upon the capacity of the ultracentrifuge rotor. Beyond this, it has been assumed that the testing would be carried out in the laboratory in which other work was in progress, so that both the operator and the equipment could be employed for other purposes when not actually in use in detecting viruses in foods.

An itemized summary of the labor required when method B is applied to a single food sample, or to a full load of 12 food samples, is presented in table 1A. It is not surprising that great economy is achieved by testing 12 samples at once. Based upon the level of skill required of the operator, and prevailing local wage rates, these operator times have been charged at $2.50 per hour. Labor consumed by the additional step in method C is approximately 3 minutes per sample.

The other costs of a testing method are much more difficult to assess accurately. Fortunately, with the exception of the special glass funnels which have been fabricated for use in the polyethylene glycol preliminary concentration, none of the equipment required is so highly specialized that it cannot be used for other purposes. In the case of the relatively large items of equipment, the costs of use have been based upon prorated depreciation plus costs of maintenance, if known. Smaller items such as reusable centrifuge tubes and bottles are charged on the basis of probable useful life plus costs of cleaning and preparation to permit them to be reused. Consumable supplies are, of course, charged at cost. A summary of these costs is presented in table 2A. These figures estimate the cost per sample when samples are being processed on a routine basis at 12 per day, and full utilization of labor and equipment is being made when it is not engaged in testing food samples. If this were not the case, one might further assume that the costs of labor and equipment (other than the tissue culture) would be doubled, so that the cost per sample would be increased from $3.88 to $6.76 per sample for method B, and from $4.43 to $7.86 for method C.

TABLE 1A

Summary of labor time consumed by various operations in the performance of method B

Operation	Minutes consumed	
	Single sample	Set of 12
Preparation of diluent	15	15
Samplings, slurrying, and centrifugation	50	120
Setup for polyethylene glycol concentration	60	120
Transfer to ultracentrifuge	5	45
Resuspension of pellet and inoculation of culture	5	30
Preparation of agar medium	15	15
Rinsing and overlaying culture	5	15
Total	155	360

TABLE 2A

Itemized estimated cost per sample of testing samples in lots of 12 by two methods

Category	Item	Cost	
		Method B	Method C
Capital equipment	Servall Omnimixer	$0.02	$0.02
	Servall RC2-B centrifuge with GSA rotor	0.05	0.05
	Spinco Model L ultracentrifuge	0.29	0.29
	Number 30 rotor for ultracentrifuge	0.06	0.06
		0.42	0.42
Minor equipment	Centrifuge bottles	0.16	0.32
	Rotor-knife for slurrying	0.06	0.12
	Funnel for first concentration	0.31	0.31
	Ultracentrifuge tube	0.02	0.02
	Miscellaneous glassware	0.20	0.20
		0.75	0.97
Supplies	Tissue culture	1.00	1.00
	Reagents and other consumables	0.46	0.66
		1.46	1.66
Labor	At $2.50/hr.	1.25	1.38
Total		$3.88	$4.43

The alternative cost of testing the food samples without concentration can also be estimated. In this case, the first assumption is that the level of contamination of the food is comparable to that at the 50% end point of the standard method, which is to say 5 PFU/25 gm. sample, or approximately 0.2 PFU/gm. of sample. One further assumes that the required quantity of food sample (in grams) would be slurried in 4 times as much buffer (in milliliters), clarified briefly by low-speed centrifugation (2,000 × g), and inoculated into the required number of flasks, using the further factor of 0.69, which the Poisson formula indicates is the number of infectious units which would have to be present per sample to yield 50% positive tests. Considering all of these factors, one arrives at the conclusion that approximately 40 flask cultures would be required per sample to achieve a comparable level of test sensitivity without concentration. This would suffice to test approximately 20 ml. of cottage cheese extract; and assuming that cultures were produced by the methods detailed in appendix I, would represent a cost of approximately $40. This estimate disregards any other costs of preparing the sample by the simplest possible method, and the possibility that tissue cultures might cost considerably more each if they were procured commercially or produced by less efficient methods. On the other hand, if one were producing his own tissue cultures entirely by the methods detailed, he would still be required to provide 480 flask cultures per day in order to test 12 food samples per day. This would necessitate sacrificing more than one rhesus monkey per day to keep pace with the demand, as well as requiring very extensive tissue culture space and facilities which might not be available. Clearly, the alternative cost of the methods developed, compared to much simpler methods which might be employed, is quite attractive if these levels of sensitivity are required. If considerably lower levels of sensitivity were tolerable, the relative economics might differ drastically.

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13. ABSTRACT

Methods developed for detection and quantitation of food-borne virus are described. Twenty-five-gm. samples of cottage cheese, contaminated with various quantities of Coxsackie virus, type A9, comprised the model system. Two of the methods presented have at least a 50% probability of detecting virus at levels below 5 plaque-forming units/25-gm. sample. Noteworthy aspects of these methods include use of a glycine-NaOH buffer (pH 8.8) containing approximately molar $MgCl_2$ as the diluent in which the sample is slurried, treatment of the slurry with Freon TF and bentonite to facilitate centrifuge clarification, and concentration of the clarified sample extract by a 2-stage process employing polyethylene glycol followed by ultracentrifugation. Virus in the final concentrate (0.5 ml.) of the sample has been detected and quantitated by the plaque technic in rhesus monkey kidney cell cultures. Time elapsed in processing the sample approaches 2 days, and the inoculated cultures may have to be observed for as long as 7 days thereafter. If these levels of sensitivity are desired, and if 12 samples per day are tested on a routine basis, the cost savings achieved by employing these methods rather than testing sample extracts without concentration may range from 75% to 90%.

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