QUANTITATIVE METHOD OF VIRAL POLLUTION DETERMINATION FOR LARGE VOLUME OF WATER USING FERRIC HYDROXIDE GEL IMPREGNATED ON THE SURFACE OF GLASSFIBRE CARTRIDGE FILTER[1]

AKIRA HOMMA* **and HERMANN G. SCHATZMAYR****

SUMMARY: Quantitative method of viral pollution determination for large volume of water using ferric hydroxide gel impregnated on the surface of glassfibre cartridge filter.

The use of ferric hydroxide gel, impregnated on the surface of glassfibre cartridge filter enable us to recover 62.5% of virus (Poliomyelitis type I, Lsc strain) exsogeneously added to 400 liters of tap-water.

The virus concentrator system consists of four cartridge filters, in which the three first one are clarifiers, where the contaminants are removed physically, without significant virus loss at this stage. The last cartridge filter is impregnated with ferric hydroxide gel, where the virus is adsorbed.

After the required volume of water has been processed, the last filter is removed from the system, and the viruses are recovered from the gel, using 1 liter of glycine/NaOH buffer, at pH 11. Immediately the eluate is clarified through series of cellulose acetate membranes mounted in a 142 mm Millipore filter.

For the second step of virus concentration, HCl 1N is added slowly to the eluate to achieve pH 3.5-4. $MgCl_2$ is added to give a final concentration of 0.05 M and the viruses are readsorbed on a 0.45 μm porosity (HA) cellulose acetate membrane, mounted in a 90 mm Millipore filter. The viruses are recovered using the same eluent plus 10% of fetal calf serum, to a final volume of 3 ml.

In this way, it was possible to concentrate virus from 400 liters of tap-water, into 1 liter in the first stage of virus concentration and just to 3 ml of final volume in a second step. The efficiency, simplicity and low operational cost, provided by the method, make it feasible to study viral pollution of recreational and tap-water sources.

THE study of viral flora in recreational and potable water, has been a matter of great concern among epidemiologists and virologists in the last years. This fact is showed by the efforts in the development of simple, efficient and economic method for detection of very low concentrations of

---

1 — Received for publication october 1, 1973.

* — Assistent-Professor, Virus Laboratories, Instituto Presidente Castello Branco (FIOCRUZ). Caixa Postal n.º 8016, ZC-24. Manguinhos, Rio de Janeiro, Brasil. Fellow from Conselho Nacional de Pesquisas.

** — Professor of Microbiology, Instituto Presidente Castello Branco, Fellow from Conselho Nacional de Pesquisas.

virus in large volumes of water ([1, 2, 3, 4, 5, 6]).

A portable apparatus for virus concentration has been described by Wallis, Homma & Melnick ([6]). By their method, inorganic and organic contaminants present in the water, are removed by cartridge filters of decreasing porosities and by an annionic resin without significant loss of virus at this stage. The water sample, after this partial purification, is added of magnesium chloride and filtered through the cartridge.

This paper presents modification in the virus adsorption system. The salt injection and the treatment with resin were ommited and the virus is adsorbed on ferric hydroxide gel impregnated on the surface of a glassfibre cartridge filter.

MATERIAL AND METHODS

1. Virus concentration system — Four cartridge filters (Commercial Filters Division, The Carborundum Company, Tell City, Indiana) of 10 inch of height and 2,5 inch of diameter with a central core of 1 inch along the height, are serially mounted and were connected with plastic tubes of 3/4 inch of diameter. They were housed in transparent plastic holders to make the following scheme (Figure I) :

a) Water clarifier system — Three cartridge filters of orlon or polyester fiber, of decreasing porosities (15 μm, 3 μm and 1 μm) were used. These filters are previously washed with water to eliminate all the detergent which might prevent virus adsorption in the subsequent stage([5]).

b) Virus adsorbent — Glass fibre cartridge filter impregnated with ferric hydroxide gel (FGFH. The filter is impregnated throught passage under 5 PSI (pounds/square inch) of 4 liters of ferric gel made by a mixture of an equal part of sodium carbonate at 0.01 M and ferric chloride at 0.05 M. All the virus experiments were done using the gel made up in the same day of experiment and also FGFH was used at the same day of its impregnation. After the impregnation of glassfibre cartridge filter with ferric hidroxide gel, it was washed with 20 liters of clarified and dechlorinated tap water to remove the excess of sodium carbonate. The FGFH is mounted immediately after the clarifiers filters.

2. Virus elution from FGFH — The recovery of adsorbed virus to the FGFH was done using glycine-NaOH buffer, pH11.0([6]). The same eluent is filtered throught the FGFH for three times. The eluate, without changing the pH, is clarified right away by filtration throughg series of cellulose acetate membranes (Millipore Corporation, Bedford, Mass.), of decreasing porosities (15 μm, 0.64 μm, 0.45 μm and 0.22 μm average pore diameter-APD) mounted in a filter of 142 mm of diameter.

3. Re-concentration of virus from the eluate of FGFH — The clarified eluate is adjusted to pH 3.5-4.0, adding slowly HCl 1N. Then Mg_2Cl is added to a final concentration of 0.05 M. The viruses, by filtration, are re-concentrated on cellulose acetate membrane of 0.45 μm APD, in a 90 mm filter.

The virus elution from the cellulose acetate membrane is done using 3 ml of glycine buffer complemented with 10% fenal bovine serum, homogenating the membrane in a micro-homogenator, for 1 minute at 50,000 rpm. (Omni-mixer, Sorvall Inc.) The homogenate is centrifuged at 30,000 g for 40 minutes and the supernate was assayed for virus.

4. Poliovirus, type I (Lsc strain) was grown in monkey kidney cells maintained with Melnick's medium B and representative harvests containing high plaque-forming unit (PFU) titers were used in this study. The virus harvests were frozen and thawed, centrifuged at 3,500 rpm for 15 minutes, and frozen again in 2 ml samples at-70°C.

5. Monkey Kidney cells-Kidneys obtained from immature vervet monkeys were trypsinized, grown, and maintained as described in details elsewhere[7].

6. Water — Tap water from the Laboratory was used for all experiments described and presented dissolved solids 460 ppm and solids in suspension 33,7 mg/1. When previous water clarification was required, it was done filtering through glassfibre cartridge filter[6].

Sodium thiosulphate ($Na_2S_2O_3$) at the final concentration of 0.1 M has been added to the water, in order to dechlorinate the sample.

7. Virus quantification — The Dulbecco technique[8] has been used, with $MgCl_2$ added to the agar overlay[9]. The plaque forming-Unit (PFU) has been used for the virus quantification.

Additional details on the methodology are described in Results in the sake of better understanding.

RESULTS

I — *Virus adsorption efficiency onto the ferric hydroxide gel*

The preliminary experiments of virus adsorption to ferric hydroxide gel was tested in tubes, using solely virus and gel.

The gel was prepared as described in Material and Methods, divided into 5 portions of 10 ml each and centrifuged at 3,000 rpm for 10 minutes. To the pellet of each tube, 10 ml of distilled water was added, to remove the excess of sodium carbonate.

To the washed pellets, 10 ml of virus suspension at different concentrations, made out in clarified and dechlorinated tap-water, was added. The complex of different gel/virus were rotate for 15 minutes. Then, all samples were centrifuged and assayed for unadsorbed virus in the supernate.

As it can be seen at Table I, the ferric hydroxide gel showed to be an excellent virus adsorbent, giving nearly 100% of virus adsorption even in those tested with a very high virus concentration.

T A B L E I

Virus adsorption efficiency on ferric hydroxide gel *

PFU/10 ml	Supernate PFU unadsorbed	Percentage of virus adsorption
1.000.000	6.000	99.4
100.000	500	99.5
10.000	40	99.6
1.000	0	100
100	0	100

* Mean values of three experiments.

II — *Virus elution from ferric hydroxide gel*

This experiment was done to stablish the ideal condition for recovery of adsorbed virus to the ferric hydroxide gel. A complex of gel/virus, containing 10,000 PFU/ml was prepared as described previously. To the pellet of these mixture, were added 10 ml of different eluents. The tubes were homogenated on a rotary agitator for few minutes and then, centrifuged and assayed for viruses in the supernate.

Table II shows several eluents used for virus recovery from the gel: distilled water, dechlorinated tap-water, saline and TRIS-HCl, pH 7.5 did not remove the viruses. Good results were achieved using eluents highly alcalines (pH 11).

TABLE II

Virus elution from the ferric hydroxide gel *

Eluents used	Adsorbed virus on ferric hydroxide gel PFU = 10.000	Percentage of virus recovery
Distilled water, pH 7	0	0
Tap-water, dechlorinated, pH 7.8	0	0
Physiologic saline, pH 7.5	0	0
Tris-HCl buffer, pH 7.5	0	0
Idem, with 10% bovine fetal serum	7.800	78
Sodium Borate buffer, pH 9	8.000	80
Idem, with 10% bovine fetal serum	8.800	88
Glycine-NaOH, pH 11	8.800	88
Idem, with 10% bovine fetal serum	9.800	98

* Mean values of three experiments.

III — *Virus adsorption to cartridge filter impregnated with ferric hydroxide (FGFH)*

The contaminants present in the tap-water might go through the clarifier filters and be retained by the virus adsorbent cartridge filter (FGFH), and then interfering or competing on virus adsorption, during the processing of large volumes of water. To eliminate such possibility, the follow experiment was performed: several FGFH were pre-treated with different large volume of water before virus adsorption. The tap-water used to threat FGFH was clarified by filtration through glassfibre cartridge filter and injected of sodium thiosulphate ($Na_2S_2O_3$, final concentration of 0.01 M), to inative chlorine present in the water. The water flow rate was approximately of 20 liters per minute.

After the treatment of FGFH with predetermined volumes of water, a suspension of 4 liters of virus (containing 4,000,000 PFU) was used to challenge the FGFH's virus adsorption capacity, using 5 PSI. The filtrate was collected and assayed for unadsorbed virus.

Table III, shows that even after the treatment with several different volumes of dechlorinated tap-water, the virus adsorption capacity of FGFH was maintained. The treatment of FGFH with 1,200 liters of clarified water and a subsequent challenge with 4 liters of known number of virus particles showed 80% of virus adsorption.

TABLE III

FGFH virus adsorption capacity after treatment with several volumes of tap-water *

Liters of tap-water used to treat FGFH before the challenge with virus	Unadsorbed virus to FGFH. Total virus used in the challenge: 4.000.000 PFU/4 liters	Percentage of virus adsorption to FGFH
Control = no treatment	40.000	99
20	40.000	99
40	80.000	98
100	120.000	97
200	400.000	90
400	600.000	85
800	800.000	80
1.200	800.000	80

* Mean values of three experiments.

IV — *Virus recovery from FGFH*

When a large volume of water is filtered throught FGFH, fine water contaminants deposits on the filter surface. This contaminants may form complex with viruses and its recovery may become difficult. This possibility was tested eluting the adsorbed virus, after the treatment with several large volumes of water.

This experiment was done adsorbing 4 liters of a virus suspension containing 4,000,000 PFU, using a constant pressure of 5 PSI. After virus adsorption, the complex FGFH/virus was treated by filtration of different several volumes of water. The tap-water used in this experiments was clarified and chlorine inactivated. After the elution procedure as described in Material and Methods, the virus content of the eluate was assayed.

The results of this test are shown at Table IV. Satisfactory percentage of virus recovery was achieved even after the treatment of FGFH/virus with 1,200 liters of water.

TABLE IV

Elution of virus adsorbed on FGFH after its treatment with several volumes of tap-water. * *Total of virus adsorbed on FGFH: 4.000.000 PFU*

Liters of tap-water used to treat FGFH after the virus adsorption	Total of virus recovery from FGFH	Percentage of virus recovery from FGFH
Control = no treatment	3.600.000	90
20	3.200.000	80
40	3.200.000	80
100	3.000.000	75
200	3.200.000	80
400	3.000.000	75
800	2.800.000	70
1.200	2.400.000	60

* Mean values of three experiments.

V — *Virus concentration from 400 liters of tap-water*

After the results of basic parameters studied in the proceding experiments, the usefulness of this method was tested, simulating a test in the field, which was done in two steps: In the first step of concentration the inicial volume of 400 liters was reduced to 1 liter. In the second step of concentration this 1 liter of eluate was reduced further to a final volume of 3 ml.

a) First step of concentration. In this step, tap-water was filtered through all the system (clarifier cartridge-filters and virus adsorbent — FGFH, as in Material and Methods, in continuous flow, injecting virus simultaneously into the system.

A suspension of 4 liters containing 600 PFU/ml, plus 1 M of sodium thiosulphate ($Na_2S_2O_3$) and 2% of phenol red was prepared. This mixture, contained in a vessel under pressure, was injected uniformly into the system so that when 400 liters of tap-water were filtered thought the concentrator, all the mixture had been injected. The injection of mixture was controlled by a millimetric valve of high precision, connected just before the first clarifier filter.

After adsorption, the virus recovery was done using 1 liter of eluent, as in Material and Methods, and the results achieved are given in the Table V. As it can be seen, 75% of originally injected virus was recovered from FGFH. It was not possible to evidenciate the presence of virus in the samples after each clarifier filter due to the high virus dilution at this stage. These samples were obtained from the monitoring valve after 50, 100, 200 and finally 400 liters of water were processed. A constant water flow rate of 5 PSI was used through the filtration. Comparable experiments using water ajusted to different pH values 5 to 7.8) showed the same results.

b) Second step of virus concentration — In this step, further reduction of volume of eluate from the first step of concentration was performed.

Using following procedures, the virus in 1 liter of eluate was concentrate in final volume of 3 ml: the eluate was clarified

TABLE V

First step of virus concentration *

PROCEDURES	PFU/TOTAL VOLUME	PERCENTAGE
Total virus in the stock solution	2.400.000	100
Water clarification in continuous flow, through:		
Cartridge filter of 15 μm porosity	—	—
Cartridge filter of 3 μm porosity	—	—
Cartridge filter of 1 μm porosity	—	—
After FGFH	—	—
Elution 1 liter of glycine-NaOH buffer, pH 11	1.800.000	75

* Mean values of three experiments.

by filtration through series of Millipore membranes, then adjusted to low pH values and magnesium salts added. Finally, the virus adsorption was performed (as in Material and Methods).

The percentages of virus involved in each operation is showed at Table VI. There was no virus loss when the liter of eluate was clarified throught the membranes or by adjusting to low pH levels. The virus adsorption to and the recovery from cellulose acetate membrane, reached 83.3% of the virus present in the eluate of the first concentration step.

TABLE VI

Second step virus concentration *

PROCEDURES	PFU/TOTAL VOLUME	PERCENTAGE
Total virus in the eluate from first step virus concentration = 1.000 ml	1.800.000	100
Clarification through, series of cellulose acetate membranes, as Material and Methods	1.800.000	100
Adjustment to pH 3.5-4.0 with HCl-1N, and $MgCl_2$ addition to final concentration of 0.05 M	1.700.000	94
Virus adsorption on the cellulose acetate membrane of 0.45 μm porosity and 90 mm diameter	—	—
Virus elution, as in Material and Methods, using 3 ml of eluent glycine-NaOH + 10% bovine fetal serum	1.500.000	83.3

* Mean values of three experiments.

DISCUSSION

The viruses, particularly enterovirus, may adsorb efficiently to the precipitate of organic and inorganic salts. After adsorption, the complex virus/gel may be separate from the original medium and the virus recovered by salt complex dissolution or eluting the virus from it using a alcaline buffer plus serum protein.

This approach is used very often for purification and concentration of different viruses using salts like calcium phosphate ([10]), cobalt chloride ([11])), aluminium phosphate and hydroxide ([12]), ammonium sulphate ([13]) and protamine sulphate ([14]).

The current study is concerned with a combination of such simple and efficient technology of virus adsorption but with volume limitations, with the cartridge filtration system, which may process several hundreds of liters in a short time ([5]). The use of the cartridge filter system allowed a clear-cut advance in water virology, due to its filtration principle. The filtration in the cartridge not only traps particles on the surface, but also throu-

ghout the total depth of the filter element. This process gives progressively finer filtration through the filter tube and provide much greater solid-retention capacity than is obtained with surface filters of the same dimension. The yarn-wound filters made of polyester or orlon fibers showed excellent qualities for water clarification.

In this previous paper, when glassfibre or cellullose acetate cartridge filter has been used as virus adsorbent, salts had to be injected to the running tap water to enhance the adsorption of viruses.

As the step of salt injection requires series of delicate monitoring it was desirable to supress this step if a good virus adsorbent which would not require salts injection could be founded.

The impregnation of cartridge filters with precipitates of inorganic salts was tried in order to achieve this goal.

In the preliminary tests, it was showed that none of several kinds of precipitates show good impregnation to the cartridge filters, rather lowering the flow rate or being washed out by the running water. One exception, however, was a gel of ferric hydroxide obtained from the mixture of sodium carbonate and ferric chloride solutions, which when tested in tubes show also excellent virus adsorption qualities (Table I).

This virus adsorption property of ferric hydroxide gel showed to be stable. No liberation of virus was evidenciated in presence of water, physiologic saline solution, or TRIS/CHl, pH 7.5 buffer. However, good virus recovery percentages could be achieved using very alcalines buffers, specially when serum was added to such eluents (Table II).

The results presented in the Table III evidenciate two importants events. The first one is related to the quality of the gel impregnation to the surface of the glassfibre cartridge filter. Using such filters, impregnated as already described, there were neither lowering of flow rates not washing out by water running. Second, it was observed excellent virus adsorption properties of such filters, even those previously treated with several hundred liters of tap-water, before the challenge with virus. These results show again that the precision winding pattern which covers the entire depth of the filter tube with hundred of funnel-shaped tunnels gives better collision between virus and the gel impregnating the fiber surfaces. By the other hand organic and inorganic contaminants, at least for the kind and volumes of water tested, were not sufficient to block virus adsorption on the sites furnished by this kind of filters.

The recovery of adsorbed virus to the adsorbent like ferric hydroxide, has been achieved using alcalines buffers in presence of organic components (Table II). However, using a buffer adjusted to very high pH (pH 11.0), was possible to recover the adsorbed virus at very satisfactory rates, even when organic components were absent. This fact allowed the second step of concentration of virus (reconcentration) on the cellulose acetate membranes.

It was already known that the presence of proteinaceous components adversely affect the virus adsorption qualities of Millipore membranes ([2, 3]). The non-viral components present usually in tap-water, seem to have interfered in just very small rate in the virus recovery in our tests when filters having already virus adsorbed on them were treated with several hundred liters of tap-water, as shown in the Table IV.

The eluent used, glycin/NaOH buffer pH 11.0, has been already used before to elute virus adsorbed to the surface of glassfibre cartridge filter ([6]).

When the complete system, clarifiers and FGFH, was tested in a experiment simulating a field collection, processing the water in continuous flow, a percentage of 75% of virus recovery was obtained in the 1 liter eluate, from the total amount of virus experimentally injected into the system. With our system as far as the volume reduction is considered 1 liter of eluate means 400 times of reduction and the virus concentration reached about 300 times (Table V).

In the absence of proteinaceous components in the eluate 1 liter, the reconcentration was done using cellulose acetate membrane as virus adsorbent, to further reduction of the volume to 3 ml, reaching at this stage 83.3% of virus recovery (Table VI).

As far as the total volume of the sample processed in two steps is considered, the reduction from 400 liters to a final volume of 3 ml, represents a concentration of about 130,000 times. The presence of 1,500,000 PFU in a final eluate of 3 ml represents the recovery of 62.5% from the original virus added into the system (2,400,000 PFU). The real virus concentration rate, from the beginning of the experiment, with a virus dilution at an input of 6 PFU/ml, to 500,00 PFU/ml into the 3 ml of the final eluate, represents a concentration of around 83,000 times.

The method here presented showed to be efficient in the conditions described and viable economically. Its use seems to us to be practical, lacking of complex monitoring systems and applicable as a portable equipment for field water sample collection.

Similar systems have been used to process sewage ([15, 16]) and sea water ([17]).

SUMÁRIO

Relata-se o emprego de um concentrador portátil, o qual se mostrou capaz de recuperar 62,5% dos vírus (Polio I, amostra Lsc) experimentalmente dispersos em 400 litros de água, os quais foram reduzidos a 3 ml.

O sistema concentrador de vírus é composto de quatro filtros-bobina, em que os três primeiros são clarificadores de porosidade decrescente e não retentores de partículas, e o último, impregnado com gel de hidróxido de ferro, adsorvedor das partículas virais presentes na água processada.

Este quarto filtro, é removido do sistema após o processamento inicial da água. Em seguida, ainda na primeira fase de concentração, os vírus são eluídos do filtro-bobina, com solução de glicina / NaOH, pH 11,0. Subseqüentemente, o eluato é reclarificado, sem redução de volume, por filtra-

ção através membranas de acetato de celulose (15 μm, 1,2 μm, 0,45 μm e 0,22 μm) montadas em série, em um suporte Millipore de 142 mm de diâmetro.

Para a segunda etapa de concentração, ajusta-se o eluato clarificado a pH 3,5 e adiciona-se cloreto de magnésio até a concentração final de 0,05M. Os vírus são a seguir adsorvidos em membranas de acetato de celulose, de porosidade 0,45 μm, em filtro Millipore de 90 mm de diâmetro, novamente recuperados por trituração da membrana em gral de vidro, em presença de 3 ml do tampão glicina / NaOH, nesta fase porém, suplementado com 10% de soro bovino fetal.

Desse modo foi possível concentrar vírus, de 400 litros de água para 1 litro na primeira etapa e apenas 3 ml de volume final na segunda etapa. A eficiência, a simplicidade e o baixo custo operacional do método recomenda-os para o estudo da poluição viral de águas recreacionais e fontes supridoras de água potável.

ACKNOWLEDGMENTS

The authors wish to express their gratitude to Drs. Graig Wallis and J. L. Melnick from the Baylor College of Medicine, Houston, Texas U.S.A. where part of this work has been done, for their help and advice. We thank also the Carborundum Company, Indiana, U.S.A. and the Pan American Health Organization, for the donation of filters and equipments used in this work.

The Conselho Nacional de Pesquisas (Brasil) provided also valuable financial support for this Project.

BIBLIOGRAPHY

1 — Shuval, H. J., Fattal, B. B., Cymbalista, S. & Goldblum, N., 1960, The phase separation method for the concentration and detection of viruses in water. *Wat. Res. Perg. Press, 3*:225-240.

2 — Cliver, D. O., 1967, Enterovirus detection by membrane chromatography. In: Transmission of viruses by the water route (G. Berg) Interscience, London.

3 — Wallis, C. & Melnick, J. L., 1967, Concentration of viruses from sewage by adsorption on Millipore membrane *Bull. Wld. Hlth. Org.* 36:219-225.

4 — Wallis, C., Melnick, J. L. & Fields, J. E., 1970, Detection of viruses in large volumes of natural waters by concentration on insoluble polyelectrolytes. *Wat. Res. Perg. Press. 4*:787-796.

5 — Wallis, C., Homma, A. & Melnick, J. L., 1972, A portable Virus Concentrator for Testing use in the Field. *Wat. Res. Perg. Press. 6*:1249-1256.

6 — Wallis, C., Homma, A. & Melnick, J. L., 1972, Apparatus for Concentrating Viruses From Large Volumes, *Jour. Amer. Wat. Works Association. 64*(3):189-196.

7 — Melnick, J. L. & Wenner, H. A., 1969, Enterovirus. In: Diagnostic Procedures for Viral and Rickettsial Infections (ed. E. H. Lennette & N. J. Schmidt) Amer. Pub. Hlth. Ass. Inc.

8 — Dulbecco, R., 1952, Production of plaques in monolayer tissue cultures by single particles of an animal virus. *Proc. Natl. Acad. Sci. 38*:747-752.

9 — Wallis, C. & Melnick, J. L., 1962. Magnesium chloride enhancement of cell susceptibility to poliovirus. *Virology 16*:122-132.

10 — Salk, J. E., 1941, Partial purification of the virus os epidemic influenza by

adsorption on calcium phosphate. *Proc. Soc. Exp. Biol. Med. 46*:709.

11 — Grossowitz, N.. Mercado, A. & Goldblum, N., 1960, A simple methods for concentration of live and formaldehyde-inactivated poliovirus. *Proc. Soc. Exp. Med. 103*:872.

12 — Wallis, C. & Melnick, J. L., 1967, Concentration of viruses on aluminium phosphate and aluminium hydroxide precipitates. In: Transmission of viruses by the water route (editado por G. Berg) Interscience, London.

13 — Lund, E. & Hedstrom, C. E., 1967, Recovery of viruses from a sewage treatment plant. In: Transmission of viruses by the water route (ed. G. Berg). Interscience, London.

14 — England, B. L., 1970, Protamine sulfate precipitation of reovirus and adenovirus for their assay in sewage and effluents. *Bacteriological Proceedings*, Absts. of the 70th Annual Meeting, American Society of Microbiology, page 194.

15 — Homma, A., 1972, Estudos experimentais da utilização de filtro-bobina para a concentração de vírus da água de esgoto. Tese de Doutoramento. Departamento de Medicina Preventiva da Faculdade de Medicina da Universidade de São Paulo.

16 — Homma, A., Sobsey, M. D., Wallis, C. & Melnick, J. L., 1973, Virus concentration from sewage-accepted to publication. *Wat. Res. Perg. Press.*

17 — Homma, A., Schatzmayr, H. G., Frias, L. A. M. & Mesquita, J. A., 1973, Virus pollution determination in Guanabara Bay (in preparation).