USE OF MICRO TECHNIQUE FOR SERUM NEUTRALIZATION AND VIRUS IDENTIFICATION

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THE MICRO-TITRATION technique for serologic tests was first described in the Hungarian literature by Takatsy and associates (*1*). Sever (*2*) modified the technique and applied it to complement fixation, hemagglutination, hemagglutination-inhibition, and metabolic-inhibition tests. Rosenbaum and co-workers (*3*) further applied the technique to the serum neutralization test. Their results indicated that serum neutralization by micro technique reduced time, material, and effort and agreed well with results obtained by conventional tissue-culture tube methods. Our application of this method to the determination of neutralizing antibody titers, in paired serums, in a group of children who had received attenuated type I poliovirus vaccine produced results similar to those of Rosenbaum and associates (*3*). We also applied this micro method to the identification of polioviruses isolated from another group of children who had received trivalent attenuated poliovirus vaccine.

Materials and Methods

Micro-titration apparatus were obtained from a commercial source (*A*) and used as described by Rosenbaum and co-workers (*3*), with one modification. Plastic disposable U-plates (*B*) were used instead of Plexiglas plates. The plates were soaked in 70 percent ethyl alcohol for 1 hour and air-dried under ultraviolet light for 2 hours before being used. This procedure eliminated the toxicity described by Rosenbaum and co-workers (*3*).

Evelyn R. Walton, microbiologist, Inez A. Stewart, biological laboratory technician, and Letha LaGalle, biological laboratory aid, Kansas City Field Station, Communicable Disease Center, assisted in the laboratory procedures.

Paired serums from 90 Waynesville, Mo., school children, 6 to 9 years of age, were tested for neutralizing antibodies. Initial serum samples were obtained the day the children were vaccinated with attenuated type I poliovirus vaccine, and the second samples 4 weeks later. Serums were separated from clots the day after collection and stored at −20° C. After the initial dilution, all serum samples were heated in a water bath at 56° C. for 30 minutes.

Virus isolation was performed on stools and pharyngeal washings of 211 Columbia, Mo., school children who had received attentuated trivalent poliovirus vaccine. The specimens were frozen at −20° C. Ten percent suspensions of stool were prepared and antibiotics added; the suspensions again were stored at −20° C. until tested. Antibiotics also were added to the pharyngeal washings, which were inoculated directly into primary or secondary Rhesus monkey kidney tissue culture. Positive cultures were harvested and stored at −20° C. until identification of viruses by the micro and macro methods.

The tissue culture used was a continuous line of monkey kidney cell strain $LLCMK_2$ (*4*). Suspensions were made from a trypsin-

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dispersed monolayer with 400,000 cells per milliliter for micro tests and 80,000 cells per milliliter for macro tests.

The diluent consisted of medium 199; growth medium was medium 199 plus 5 percent chicken serum. All media contained penicillin (200 units/ml.), streptomycin (200 μg./ml.), and amphotericin B (1 μg./ml.).

We used a pool of poliovirus type I (Brunhilde strain) in both the micro and macro tests. The infectivity dose in the micro tests ranged from 80 to 250 $TCID_{50}$ and in the macro tests from 32 to 320 $TCID_{50}$.

Immune rabbit serums with antibody titers of greater than 1:1,024 to poliovirus types I, II, and III were used in both methods for identification of viruses. Four pools of poliovirus antiserums were used in the following combinations: types I and II, I and III, II and III, and I, II, and III. With the macro method, each type was used at 1:4 dilution in the combination of two antiserums and at 1:6 dilution in the combination of three antiserums. With the micro method, each type was used at 1:160 dilution in the combination of two types and 1:240 dilution in the combination of three types.

Micro technique. Serum antibody titers to poliovirus type I were determined in duplicate in micro-titration plates. Micro pipettes were used to drop 0.025 ml. of diluent (medium 199) into each well except the first row in the micro-titration plate. To the wells of the first row, about 0.1 ml. of serum, diluted 1:4 in medium 199, was added by means of 1-ml. disposable-plastic pipettes. Then 0.025 of this dilution was transferred by micro-titer loops to the next row of wells to give a dilution of 1:8. Similar serial loop transfers gave a final dilution of 1:4,096. One row of wells with a dilution of 1:8 served as serum toxicity controls. Next, 0.025 ml. of poliovirus type I diluted to 1×10^{-4} (about 100 $TCID_{50}$) was added to all wells except the serum toxicity controls, which received the same amount of diluent. The plates then were placed in a humidified incubator in an atmosphere of about 5 percent CO_2 in air at 37° C. for 30 minutes. Subsequently, an additional 0.025 ml. of diluent was added to each well. (This step eventually was eliminated by incorporating this volume in cell suspension.)

A cell suspension was prepared in growth medium with an initial pH of 7.0. Carbon dioxide was bubbled through the cell suspension until an orange color (pH about 6.8) was obtained before it was dispensed into the wells. Approximately 10,000 cells, contained in 0.025 ml. of growth medium, were dropped into each well. Finally, each well was overlaid with approximately 0.10 ml. of sterile mineral oil. This amount of mineral oil, used to insure no drying of the wells, produced no toxicity when autoclaved at 15 pounds for 15 minutes. The plates were incubated in a humidified CO_2 incubator at 37° C. Infectivity titers of poliovirus type I were prepared at tenfold dilutions in tubes; 0.025 ml. of each appropriate dilution was dropped into eight wells, which were otherwise treated as outlined.

The tissue cultures were observed with an inverted microscope, and the cytopathogenic effect (CPE) was recorded as 1+ to 4+ (25–100 percent) on the basis of the extent of cytolysis. Twenty-five percent or greater cytopathogenic effect indicated infection. The test was read and recorded 48 hours after the test dilution of poliovirus type I (100 $TCID_{50}$) revealed 2+ CPE. Inhibition of 1+ CPE was considered to be protective, and the highest dilution showing this inhibition was recorded as the neutralizing antibody titer. Serums were retested if the duplicate results did not agree within one twofold dilution. No attempt was made to compare color change with the cytopathogenic effect since our initial observations did not reveal a consistent correlation. Furthermore, the CO_2 in the incubator might have an effect on lowering the pH.

Identification of viruses from stool and pharyngeal specimens was accomplished with harvests from positive primary or secondary Rhesus monkey kidney tissue cultures. Each harvest was diluted 1:100 in medium 199, and about 0.025 to 0.030 ml. was added to each of 12 wells with disposable 1-ml. pipettes, calibrated in hundredths. To each well, 0.025 ml. of diluent also was added. To the correct well, 0.025 ml. of the appropriate antiserum mixture (poliovirus types I, II, and III, I and II, II and III, and I and III) was added; 0.025 ml. of diluent also was added to the accompanying virus control. The micro-titration plates were incubated at 37° C. for 30 minutes. Then 0.025

Table 1. Comparison of poliovirus type I neutralizing antibodies in 90 paired serums by micro and macro techniques

Agreement of techniques	Serum 1		Serum 2		Total	
	Number	Percent	Number	Percent	Number	Percent
Within twofold dilution	86	96. 6	83	93. 3	169	94. 9
Within fourfold dilution	3	3. 4	6	6. 7	9	5. 1
Total	[1] 89	100	[1] 89	100	178	100

[1] Inconsistent results obtained for 1 pair of serums by both techniques.

ml. of $LLCMK_2$ cells and 0.10 ml. of mineral oil were added to each well.

Thus each virus or virus mixture to be identified was represented by 4 wells for virus control and 3 for each of the 4 antiserum mixtures, for a total of 12 wells. The test was read 48 hours after the appropriate virus control showed 2+ CPE. Identification of the isolate was recorded only if the cytopathogenic effect was inhibited by the appropriate antiserum.

Macro technique. Neutralizing antibodies for poliovirus type I were determined for the same serum samples, by conventional tissue culture methods, in tubes. $LLCMK_2$ tissue culture was grown in roller tubes in medium 199 plus 5 percent calf serum and changed on the second day to maintenance media consisting of medium 199 plus 1 percent chicken serum. The serums were diluted twofold in phosphate-buffered saline. Equal amounts of virus (about 100 $TCID_{50}$) and the appropriate serum dilution were incubated for 60 minutes at 37° C. before 0.2 ml. of the mixture was inoculated into each of two tubes. The tubes were read and the results interpreted as described for the micro procedures.

Identification of isolates from stool and pharyngeal specimens was accomplished with 1:100 dilution of the harvests used in the micro tests. The virus dilution was incubated with equal amounts of the appropriate antiserum mixture (poliovirus types I, II, and III, I and II, II and III, and I and III) at 37° C. for 60 minutes. Then 0.1 ml. of this mixture was added to each of three tubes of secondary Rhesus monkey kidney tissue culture, which had been grown in Hank's medium with 0.5 percent lactalbumin hydrolysate and 2 percent calf serum and changed to Eagle's maintenance medium (*5*) on the third day. The tubes were read and the results interpreted as described for the micro tests.

Results

Ninety pairs or 180 serum samples were tested by both the micro and macro methods for neutralizing antibody titers to poliovirus type I. The results are compared in table 1. Inconsistent results were obtained for one pair of serums tested repeatedly by both methods. The results for the remaining 89 pairs agreed within one twofold dilution in 169 of 178 serums, or 94.9 percent. Nine serums showed that titers differed by more than a twofold but less than a fourfold dilution. None differed by more than a fourfold dilution. The geometric mean titer for the first serums was 39 and for the second serums 436 by the micro method. By the macro method, the geometric mean titers were 34 for the first serums and 400 for the second serums. A fourfold or greater antibody rise

Table 2. Identification of 78 isolates from pharyngeal and stool specimens by micro and macro techniques

Isolates	Identification		Total
	Agreement	Disagreement	
Poliovirus I	35	0	35
Poliovirus II	3	0	3
Poliovirus III	2	0	2
Poliovirus I and II	14	0	14
Poliovirus II and III	0	0	0
Poliovirus I and III	2	0	2
Poliovirus I, II, and III	20	2	22
Total	76	2	78

to poliovirus type I was shown in 72 serums, or 80 percent, by the micro method and 73 serums, or 81 percent, by the conventional macro technique.

Seventy-eight isolates from pharyngeal and stool samples were identified by both the micro and macro methods, with complete agreement in 76 of the 78 isolates. The various mixtures were identified as indicated in table 2. One stool-specimen isolate was identified as poliovirus type II and III mixture by the micro method and I and III by the macro method. The second specimen was identified as a poliovirus type I, II, and III mixture by the micro method and I and II by the macro method. The two isolates were retested by both methods with virus dilutions of 1:10, 1:100, and 1:1,000, and both contained all three poliovirus types at the 1:10 dilutions.

Discussion

The micro method has proved to be of great value in various serologic procedures, with many advantages over the standard tube method. It reduces time, expense, material, and effort. Furthermore, a considerable saving of specific antiserums, stock virus, tissue culture, and tissue culture reagents is effected. Many types of antibodies can be titered with a relatively small amount of serum, thus preserving this precious commodity for further study.

Rosenbaum and co-workers (*3*) reported a comparison of poliovirus type III neutralizing antibodies in 56 serums (28 pairs) using the micro and macro methods. Agreement was within a twofold dilution in 49, or 87.5 percent, of the serums. Our results showed similar agreement in comparing the two methods for measurement of poliovirus type I neutralizing antibodies.

We have also adapted the micro method for the identification of poliovirus. Comparison of the results with those obtained by the standard tube method indicated 97 percent agreement. The difference in two specimens was thought to be caused by the presence of a small amount of infectious particles of one of the polioviruses at the dilution used. This was confirmed by retesting the isolates at a lower dilution, and the presence of all three polioviruses was confirmed by both methods. It would seem that other viruses, especially enteric viruses, might also be identified by this method, with a saving of specific antiserums, although such tests have not been made in our laboratory.

Summary

Ninety pairs of 180 serum samples were tested in our laboratory for type I poliovirus neutralizing antibodies by both the micro and standard macro methods. The titers agreed within a twofold dilution in 95 percent of the samples and within a fourfold dilution in the remainder. Viral isolates from stool specimens and pharyngeal washings were identified as polioviruses by both methods. These isolates were found to contain mixtures of polioviruses. There was 97 percent agreement between the two methods.

The micro technique offers several advantages: saving of time, expense, material, effort, specific antiserums, stock virus, tissue culture, and tissue culture reagents. Perhaps most important, many types of antibodies can be titered with a relatively small amount of serum, thus preserving this precious commodity for further study. Although not investigated in our laboratory, it would seem likely that this technique can readily be applied to other virus systems.

SUPPLY REFERENCES

(*A*) Microtiter, Cooke Engineering Co., Alexandria, Va.

(*B*) Plastic items, Falcon Plastic Co., Los Angeles, Calif.

REFERENCES

(*1*) Takatsy, G. Y., Furesz, J., and Farkas, E.: Studies on the quantitative relationship of influenza virus antibody union by means of a simple antibody absorption test. Acta Physiol Acad Sci Hung 5: 241–254 (1954).

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(*4*) Hull, R. N., Cherry, W. R., and Tritch, O. J.: Gross characteristics of monkey kidney cell strains LLC-MK$_1$, LLC-MK$_2$, LLC-MK$_2$ (NCTC–3196) and their utility in virus research. J Exp Med 115: 903–917, May 1962.

(*5*) Eagle, H.: Amino acid metabolism in mammalian cell cultures. Science 130: 432–437, Aug. 21, 1959.

PHR SYNOPSES

HOROWITZ, HERSCHEL S. (Public Health Service), LAW, FRANK E., and PRITZKER, THEODORE: *Effect of school water fluoridation on dental caries, St. Thomas, V.I. Public Health Reports, Vol. 80, May 1965, pp. 381–388.*

Since the fall of 1954, fluoride has been added to the water supply of the Lincoln School in the town of Charlotte Amalie, St. Thomas, V.I., a community with fluoride-deficient sources of drinking water. To compensate for exposure of the children to fluoridated water at school but not at home and for their age when first exposed, a fluoride level of 2.3 ppm was postulated as a suitable level for investigation. Analysis of 96 water samples taken during 8 school years indicated an average school-year fluoride level of 2.34 ppm. However, the water was sampled most frequently after the fluorides were added or just after the equipment was serviced or repaired, and this figure therefore may represent an overestimation of the actual levels of fluoride maintained during the study period.

A dental survey conducted in the fall of 1962 showed that the caries level of children who had attended only the test school was substantially lower for all but one grade than the level of children who attend other comparable schools in the same community where the water was fluoride deficient. The difference of 21.9 percent for all children in the test school in their mean proportion of teeth affected by dental caries was significant at a probability level of less than 0.001.

WEINERMAN, E. RICHARD (Yale University School of Medicine and Grace-New Haven Community Hospital, New Haven, Conn.), RUTZEN, S. ROBERT, and PEARSON, DAVID A.: *Effects of medical "triage" in hospital emergency service. Yale studies in ambulatory medical care. Public Health Reports, Vol. 80, May 1965, pp. 389–399.*

The twin trends of rising patient load and the increasing proportion of nonurgent conditions have strained the resources of hospital emergency services. Important causes, among many others, are the reliance of the lower economic groups on this source for general medical care and the use of emergency facilities by all segments of the community as a substitute for unavailable private care.

In the effort to adapt its program to these changes, a new screening and referral procedure termed medical "triage" was instituted at the Grace-New Haven Community Hospital, New Haven, Conn. Continuous evaluation of its impact and of additional service needs has been undertaken, with initially encouraging results.

Specific studies of disposition patterns, clinic referrals, and staff attitudes toward the innovation are reported. Primary emphasis has been on the methodology of research in the emergency room setting. Findings indicate that the triage process does not appreciably alter the distribution of dispositions, that appointments made for clinic referrals are kept by the expected proportion of patients, and that staff reactions are essentially positive and cooperative.

LIGHT, HAROLD (Gouverneur Ambulatory Care Unit, Beth Israel Hospital), and ROSENTHAL, JESSE: *Pilot study of quality and standards in filling spectacle prescriptions. Public Health Reports, Vol. 80, May 1965, pp. 401–404.*

A study was conducted of the quality of spectacle prescriptions dispensed to indigent patients of the Gouverneur Ambulatory Care Unit, Beth Israel Hospital, New York City. An optometrist checked the spectacles against a duplicate of the prescription, inspected lenses and frames for imperfections, and rated adjustment and size of frame.

During the 6-month study period 123 inspections were made. More than 50 percent of all spectacles supplied through three different sources were rejected. Errors included cylinder axes, power, vertical prisms, size of frame, and distance between lens centers. Frequently spectacles were not case hardened as specified.

In general, the results indicated an incompatibility between the spectacles dispensed and the conventionally accepted standards which were applied in checking them. This would seem to indicate a need for revision of the standards or assumption of increased supervisory responsibility by the health agency supplying the prescriptions, or both.

PHR SYNOPSES

BAHN, ANITA K. (Public Health Service), GORWITZ, KURT, KLEE, GERALD D., KRAMER, MORTON, and TUERK, ISADORE: ***Services received by Maryland residents in facilities directed by a psychiatrist: First year of a State case register. Public Health Reports, Vol. 80, May 1965, pp. 405–416.***

Data from the first year's operation of a statewide psychiatric case register in Maryland were used as the basis for a variety of measures related to psychiatric care, such as unduplicated counts of individuals receiving psychiatric services, correction factors for computing admission rates and prevalence, combined inpatient and outpatient admission rates, 1-year-prevalence ratio, and number of episodes and days of hospital and clinic care per person per year by such patient variables as age, sex, color, place of residence, and type of facility. The data did not include reports from private psychiatric practice or from community mental health agencies not under psychiatric direction.

A statewide psychiatric case register can provide statewide and community data for the mental health program planner and administrator on the psychiatric services received by patients during a given 1-year period.

HAYMAN, C. R. (Pennsylvania Department of Health), BOCK, H. B., TURNBULL, C., and PETTIS, G. S.: ***Oral poliomyelitis vaccination program in Berks County, Pennsylvania. Public Health Reports, Vol. 80, May 1965, pp. 417–422.***

Detailed tabulation and analysis of all registrations were accomplished for the oral poliomyelitis vaccination program conducted by the medical society of Berks County, Pa., in 1963.

Including nonresidents, 171,256 persons received at least 1 type of vaccine. Sixty percent of the county's residents participated, 3 percent receiving 1 type, 8 percent 2 types, and 49 percent all 3 types. The proportion receiving all three ranged from 12 percent of persons over 65 years to 86 percent of school children. Protection by the three types was lower in Reading than in the rest of the county, and in that city lowest rates prevailed in the lowest socioeconomic area and among nonwhites.

Sixty-five percent of the residents of Berks County are estimated to be well protected by Salk, Sabin, or both. The Sabin program, however, needs to be repeated for infants throughout the county and for preschool children in many areas, especially in southwest Reading. Moreover, in all communities, even where programs have been completely successful, continuing immunization of oncoming generations of children during their first year of life is necessary.

PIPER, GERALDINE M. (Public Health Service), FRANK, BERNARD, and THORNER, ROBERT M.: ***Survey of home-delivered meals programs. Public Health Reports, Vol. 80, May 1965, pp. 432–436.***

A preliminary study by the Division of Chronic Diseases, Public Health Service, of operating home-delivered meals programs emphasizes the fact that the services are available to small numbers of people in scattered communities. Sixteen of the 22 services that reported on their program activities submitted individual records for 439 persons.

The survey indicates that a typical meals on wheels program is a community service most often located in a metropolitan area in the eastern part of the United States. Service probably was initiated shortly before or sometime in 1960. The average number of patients served is approximately 20 to 40 persons per day, with the midday and evening meals being delivered 5 days a week, Monday through Friday. The program has the professional services of a dietitian, nutritionist, or home economist; the meals are prepared in the agency kitchen; and modified diets are provided. Volunteer vehicles transport the meals to the recipient's home, and a fee for the service is charged according to the person's ability to pay.

The type of person most often found to be receiving meals on wheels service is a woman in her seventies who lives alone but is not necessarily homebound. She is ambulatory, can feed herself, and is sound of mind. She either owns or shares an apartment or house and has her own kitchen facilities. Social Security, savings or investments, or Old Age Assistance or a combination of these is her source of support. It is quite probable that she or a friend or neighbor initiated the referral for the service.

PHR SYNOPSES

LEEDOM, JOHN M. (Public Health Service), GRAHAM, ALBERT C., and BYER, M. A.: *1963 epidemic of poliomyelitis in Barbados, West Indies. Public Health Reports, Vol. 80, May 1965, pp. 423–431.*

From March 23 through June 19, 1963, 68 cases of paralytic poliomyelitis occurred in Barbados, the island's first epidemic in 30 years.

Poliovirus type I was isolated from three patients with paralytic disease. The age distribution of the patients, with illness concentrated in the 0–5 year age group, was typical of that usually encountered in a developing tropical country. Only one case occurred in a member of the upper socioeconomic groups, perhaps reflecting the use of inactivated poliovirus vaccine by this segment of the population.

Enterovirus carriage studies on June 4 and 5, near the peak of the epidemic, showed that 6 of 46 (13 percent) well children aged 1–3 years harbored type I poliovirus, indicating moderately widespread seeding of this agent in small children. Concomitant studies of antibody prevalence in serums from 22 of these children revealed that 55 percent lacked antibodies to poliovirus type I.

The epidemic terminated abruptly within 6 days of a mass feeding of oral poliovirus vaccine in which some 27 percent of the 0–5-year-olds were fed within a 3-day period. It is believed that the mass vaccine feeding was instrumental in effecting an early termination of the epidemic.

LASERSOHN, WILLIAM (Saint Luke's Hospital, Cleveland, Ohio): *Acute diarrheal diseases in a Zuni community. Public Health Reports, Vol. 80, May 1965, pp. 457–461.*

A study was made of 582 episodes of diarrhea seen at the Public Health Service Indian Hospital, Zuni, N. Mex., between June 1, 1961, and October 31, 1962. No pathogens were cultured from specimens taken in 59 percent, or 343 episodes. Of the 239 episodes in which pathogens were found, *Shigella* was isolated in 173; *Escherichia coli* in 68; and *Salmonella* in 17. *E. coli* and *Shigella* were cultured from the same stool specimen in 20 of the episodes.

All age groups were included in the study. The most susceptible age group was those under 5 years. Most illnesses were benign and self-limited. Unusual severity indicated a *Salmonella* infection.

Antibiotics were used in treating the patients. The sulfonamides were relied upon most heavily. The triple sulfonamide (sulfadiazine, sulfamerazine, sulfamethazine) and furazolidone were used in a large number of cases and showed similar results, except that furazolidone-treated cases had a higher proportion of persistently positive cultures. Sulfisoxazole appeared to be unusually effective, though used only six times. There were two deaths, both the result of severe dehydration.

LAMB, GEORGE A. (State University of New York), PLEXICO, KATHRYN, GLEZEN, W. PAUL, and CHIN, TOM D. Y.: *Use of micro technique for serum neutralization and virus identification. Public Health Reports, Vol. 80, May 1965, pp. 463–466.*

Ninety pairs or 180 serum samples were tested in our laboratory for type I poliovirus neutralizing antibodies by both the micro and standard macro methods. The titers agreed within a twofold dilution in 95 percent of the samples and within a fourfold dilution in the remainder. Viral isolates from stool specimens and pharyngeal washings were identified as polioviruses by both methods. These isolates were found to contain mixtures of polioviruses. There was 97 percent agreement between the two methods.

The micro technique offers several advantages: saving of time, expense, material, effort, specific antiserums, stock virus, tissue culture, and tissue culture reagents. Perhaps most important, many types of antibodies can be titered with a relatively small amount of serum, thus preserving this precious commodity for further study. Although not investigated in our laboratory, it would seem likely that this technique can readily be applied to other virus systems.

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