

STUDIES ON LABORATORY DIAGNOSIS
OF VIRAL INFECTIONS

by

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ABSTRACT

This study was initiated to examine methods of improving virus diagnostic rates in view of the presently low results obtained by the routine laboratory. First, comparisons of diagnostic rates were made between a routine laboratory and a special investigation at the Family Practice Unit (FPU) Ottawa Civic Hospital, where specimens were inoculated immediately into tissue cultures. Both virus isolation rates and serologic diagnosis were about twice as high in the FPU as were obtained in the routine laboratory. Results could also be obtained earlier. Overall diagnostic rate in the FPU was 33.3% but 12% in the routine laboratory. The effects of storage at room temperature on the sensitivity of primary African Green Monkey Kidney (pAGMK) and Vero cells were studied with the view of using such a technique for storage of cells by satellite hospitals and wards ready for immediate inoculation. The sensitivity of pAGMK to herpes simplex and coxsackie B₄ viruses was found to be affected after storage at room temperature for 4 days. Sensitivity of Vero cells did not seem to be affected under our experimental conditions. However virus susceptibility tests showed that pAGMK cells were more versatile and as such could still be used together with Vero cells in such storage procedure for virus isolation. Direct microhemagglutination with throat washing was done to determine the usefulness of such a technique in rapid diagnosis of influenza virus infection. The high percentage of false positives obtained threw some doubt on the application of such a method for virus diagnosis.

Because stool specimens usually contain low concentration of viruses, a concentration method with polyelectrolytes was examined for adapta-

tion for the routine laboratory. The results confirmed the usefulness of this method and a reasonable simple method of concentrating viruses from stool is described. Such a method could rid stool specimens of their toxic constituents through selective adsorption of viruses and could be used for other specimens like cerebrospinal fluids and autopsy materials which are usually toxic for tissue culture cells.

Comparison was also made between venous and capillary blood for complement fixing (CF) antibody titres and immunoglobulin levels. Higher values were found in venous blood. The implications of these findings in paediatric diagnostic procedures require that further detailed study should be done.

GENERAL INTRODUCTION

Advances in science and medicine have led to the recognition that many human diseases are caused by viruses. Horsfall (1965) reported that about 60% of all episodes of illness are attributable to viral infections. This recognition has made the physician look to the virologist for more meaningful laboratory diagnosis of viral diseases.

Until recently, there has been a lot of antipathy on the part of the physician and to a certain extent, the patient towards laboratory diagnosis of virus infections. This is because there are not many viral chemotherapeutic agents. Also virus diagnosis is time consuming and by the time the results are obtained the patient "is either dead or on his way to recovery."

The situation is however changing. Chemotherapeutic agents are now available for selective cases. Cytosine arabinose and iodo-deoxyuridine are being used in topical and systemic treatment of herpes simplex infections (Buthala 1964, Tomlinson and MacCallum 1970). Amantadine is reported to have prophylactic as well as curative effect on influenza infection (Galbraith et.al. 1969). Methisazone has been shown to provide protection from smallpox. (Floor-Wieringa et.al. 1967) Many more agents are currently being investigated for their potential use. It seems possible that within the next decade virus chemotherapy could be a reality.

Rapid methods for diagnosis are being developed in order to provide results within the shortest possible time, ideally during the acute phase of illness. Direct examination of specimen is one of the methods being used. Kalter (1950) reported a rapid method for detection of influenza virus during

epidemics by screening throat washings by direct hemagglutination (HA) test. Peizer (1965) and Doane et.al. (1967) also reported the identification of influenza and parainfluenza respectively from throat washing and nasopharyngeal secretions by HA. Many workers both in the past and the present have examined nasal smears from patients by immunofluorescence for the identification of viral infections (Liu 1956; Tateno et.al. 1962; Ebisawa et. al. 1968 and Gardner et.al. 1970).

With the greatly increased availability of the electron microscope as a laboratory tool, it is being used in the rapid diagnosis of virus infections. This seems to offer a lot of promise for the future. (Doane et.al. 1967; 1969; Joncas et.al. 1969; and Anderson and Doane 1970). There are however not that many clinical specimens which can be examined with great success by the direct examination procedures since the concentration of viruses are not high enough to permit such exercise. Specimens therefore need to be concentrated. The use of polyelectrolytes (PE60) for concentration of viruses from sewage and stool had been reported by Wallis et.al. (1969; 1971). The use of ultracentrifugation is one of the old methods still in use. Specimens are also inoculated into tissue cultures and these are examined by the electron microscope for virus particles. More sensitive cells are being sought for isolation and identification of viruses (Cashon et.al. 1970).

Proper collection and handling of specimens is highly important to the successful recovery of any agent which might be present. Holzel et.al. (1963) reported of a system whereby specimens were inoculated directly into

tissue cultures by the medical officer at the receiving room. They noticed an increase in the isolation rates of viruses. A report by the Manchester Public Health Laboratory (1964) once again indicated higher isolation rates when specimens were inoculated directly or immediately into tissue cultures. Their report showed that isolation rates from throat swabs by this method were 5% higher than isolations from stored specimens even when these have been held frozen at -30°C . Herrmann (1967) suggested that specimens should be inoculated immediately after they were collected to enable virus isolation within the shortest possible time. Rose (1969) reported that even for relatively thermostable adenoviruses, the best results were usually obtained when unfrozen specimens were inoculated into tissue cultures within 6 hours after collection.

It was within this framework of reference that the present study was undertaken to determine the effectiveness and usefulness of a Virus Diagnostic Service working in close collaboration with clinicians in community medicine; and also to examine methods of providing rapid as well as higher diagnostic rates than those obtained presently.

We therefore examined the following:-

- 1) that immediate inoculation of specimens into tissue cultures gives higher virus recovery from specimens. Cell cultures were supplied to the Family Practice Unit, Ottawa Civic Hospital for immediate inoculation with specimens and diagnostic rates with this system was compared to that in a Routine Laboratory. Primary African green monkey kidney (pAGMK) and Vero cells were

also examined for their durability and sensitivity to various viruses, after these cells have been maintained at room temperature.

- 2) significance of direct haemagglutination with throat washing as an early "signal" of possible influenza-like upper respiratory tract infection was investigated. These specimens were also examined by electron microscopy.
- 3) adaptation of the PE 60 concentration method described by Wallis et.al. (1969) for routine laboratory use and
- 4) comparison between venous and capillary blood for CF antibody titres and immunoglobulin levels by immunodiffusion were also studied.

L I T E R A T U R E R E V I E W

LITERATURE REVIEW

I: VIRUS ISOLATION

Isolation of viruses from clinical specimens is done by inoculation of susceptible hosts such as animals, hen's embryonated eggs and tissue cultures. The use of animals for virus isolation has many disadvantages. Inoculation of an animal with specimen may elicit immune responses which may interfere with the growth of the virus. Also diagnosis is based on whether the inoculated animals would show evidence of infection which depends on whether the animal is susceptible to the virus. The expense involved in maintaining animals also makes such a host system less attractive for routine use. Immune responses and host susceptibility to the virus once again may become a problem when embryonated eggs are used for virus isolation. With tissue culture however the individual cell is the host for virus and complications of immune responses can be eliminated easily. Cell cultures are easier to maintain, requiring less space than animals or even eggs and less attention than animals. Specimens from patients can be inoculated immediately into tissue culture tubes which can be stored for periods at 37°C. On the other hand, specimens can be inoculated immediately into embryonated eggs only if there is an almost daily supply of eggs to the laboratory since these have to be kept at 37°C for specified periods before inoculation, to achieve optimal virus recovery. Improvements in cell culture techniques, availability of many variety of cells of different origins, and their wide range of susceptibility have made this system the prime host-cell system for the isolation of viruses.

In vitro tissue cultures were first employed in 1913 for the propagation of vaccinia virus (Steinhardt, 1913) and were successfully utilized for preparation of vaccines against yellow fever (Lloyd, et.al., 1936). Later, Weller and Enders (1948) described a technique for the cultivation of mumps virus in tissue cultures. It was however the report of Enders, Weller and Robbins (1949) that the Lansing strain of poliomyelitis virus could be grown in various human embryonic tissues (arms, legs, and intestines) of non neural origin, which stimulated extensive use of cell culture systems for the study of human and animal viruses. Cells of many origins have been used since then and attempts would be made to review some of these which are in common use.

Cell cultures may be divided into several types based on certain characteristics of the cells. Primary cells consist of cells freshly explanted from tissues, cell strains and cell lines would be as defined by Hayflick and Moorhead (1961) A cell strain is therefore a population of cells from animal tissue subcultivated more than once in vitro and which lacks the property of indefinite serial passage while preserving the chromosomal karyotype of the original tissue. A cell line is a population of cells from animal tissue grown in vitro by serial subcultivation for indefinite periods of time with a departure from the chromosome number characterizing its source.

(A) Tissue Cultures

(i) Human Amnion Cell Cultures: The amnion offers a relatively large source of normal human tissue which can be obtained readily from many hospitals. The use of such tissue for the growth and assay of viruses was first reported by Ziteer, et.al. (1955).

Cells of the full-term amnion were found to be better than those derived from amniotic membranes of 2 or 3 months gestation. These authors were able to cultivate cells of amnion tissue in plates, tubes and flasks and these cells grew well in media containing sera from human, cow, horse, ox and lamb, and were found to be susceptible to poliomyelitis viruses. The amount of virus produced by the cultures measured as plaque forming unit on monkey kidney cells was the same as was usually obtained from monkey kidney cells. These cells provided a suitable alternative to monkey kidney cells for large-scale production of poliomyelitis virus.

Weinstein et.al. (1956) reported on the susceptibility of primary amnion cells to a variety of viruses. They confirmed the results of Ziteer et.al. (1955) that polioviruses could be grown on amnion cells. Other viruses namely adenoviruses 1 to 8, herpes simplex, encephalomyocarditis virus (EMC), Coxsackie B3 and A9 were found to produce cytopathic effect (CPE) on primary amnion cells; no CPE was produced when these cells were inoculated with influenza A (PR8) and influenza B, Coxsackie B2 and B4 and Newcastle disease virus. Dunnebacke and Ziteer (1957) described an improved and modified procedure for cultivating such cells. Their experience was based on cultivation of over 400 membranes. They reported that the membrane could be kept in phosphate buffer at room temperature up to 12 hours after delivery. They studied different media to determine optimal growth conditions of the cells.

Medium 199 and Earle's Balanced Salt solution (BSS) containing 0.5% lactalbumin and 0.1% yeast extract were used. They found that Medium 199 supported growth more frequently than did Earle's BSS; human and lamb sera gave equally good results whereas ox and horse sera were less effective. Although Medium 199 with varying percentage of human serum (5, 10 and 20%) was effective, the cells grown in 5% serum did not withstand agar overlay satisfactorily.

Takemoto and Lerner (1957) reported a similar susceptibility range as did the other workers and added that mumps and dengue type 1 did not produce CPE on amnion cells. They were however able to get a partial cell degeneration with Coxsackie B2 on their strain of amnion cells. Primary human amnion cell cultures were found suitable for primary isolation of adenoviruses, polio and herpes simplex viruses. Fogh and Lund (1957) reported continuous cultivation of cells from human amniotic membrane. Weller et.al. (1958) reported changes induced by varicella virus in a cell line of human amnion cell cultures. Primary isolations of measles virus had been reported using amnion cells (Enders, 1962; Gresser and Chang, 1963). Weller and Neva (1962) reported the isolation of rubella virus in amnion cell cultures with production of CPE and this cell represents one of the few primary cells in which rubella is known to produce CPE.

(ii) Human Fetal Kidney Cell Cultures: In 1957, Pelon et.al. reported the cultivation of human fetal kidney cells for the iso-

lation of rhinoviruses. Their success in this field stimulated many workers attempting to isolate the common cold virus to use human tissues in their studies. Tyrrell et.al. (1960) described techniques for the growth of kidney cells and their usefulness in virus isolations. Kidneys from therapeutic abortions performed between 3 and 5 months were used. Later on Tyrrell and Parson (1960) described virus interference and cytopathic effect of rhinoviruses on primary human kidney cells. Enders (1962) reported that isolation of measles viruses was most successful on primary human kidney cells. Since kidney tissues are not as readily obtainable as amnion tissues, Hayflick and Moorhead (1961) and Schmidt et.al. (1965) experimented on developing kidney cell strains. Studies were conducted using human fetal cell strain in primary isolation of human viruses and cell susceptibility tests. The results indicated that human diploid kidney cells were not as sensitive as primary monkey cells for the isolation of polio and coxsackie viruses. On the other hand human diploid kidney cells were found to be more sensitive than rhesus monkey kidney cells for the isolation of adenoviruses, and echoviruses. Other viruses like reoviruses and herpes simplex were found to be readily isolated from clinical specimens using human diploid kidney cells.

(iii) Human Fetal Lung Cell Cultures: Cultivation of normal human lung cells for virus studies began almost at the same time as that for kidney cells. Many investigators applied themselves to

establishing cell strains from fetal lung tissues. Only limited success was obtained by most of the workers until Hayflick and Moorhead (1961) reported their work on serial cultivation of human diploid cell strains. Since then their names have been synonymous with diploid lung cell strains and their methods have proven successful in many hands. Initial isolation of primary cells was done by either trypsinizing tissues or mincing these by cutting in petri dish with scissors or paired scalpels. Cells were then grown in Eagle's medium in Earles BSS, supplemented with 10% calf serum. They reported that fetal tissue could be kept for at least 5 days at room temperature or 5°C in growth medium and such treatment did not affect its viability. Primary cultures were subcultivated as soon as these were fully sheeted. The cells were trypsinized and the dislodged cells were spun, resuspended in growth medium and split up for further cultivation. In this way they were able to cultivate cells for many generations. The authors demonstrated that there was a limit to the number of subcultures that could be achieved from primary cultures. In their experience this period consisted of three phases:- phase one comprised of the time when the primary cells were establishing themselves in tubes or bottles and terminated with the formation of first confluent sheet. Phase two was characterised by luxuriant growth and during this phase cell alteration occurred. This phase might continue till about the 50th passage level when phase

three began and this was characterised by loss of cells thereafter. Cells could be preserved by freezing at any passage level during phase two and these could be recovered without recourse to special procedures.

Virus susceptibility of serially cultivated lung cells has been found to be very broad (Hayflick and Moorhead, 1961; Embil and Faulkner, 1964; and Trlifajova, et.al., 1970) and have been used to cultivate enteroviruses, adenoviruses, influenza and herpes simplex viruses; lung cells are however insensitive to coxsackie B viruses.

(iv) Other Human Cell Cultures: Cells of both malignant and non malignant origin cultivated in continuous passage have found widespread use in both diagnostic and research virology.

Gey, et.al. (1952) reported the continuous cultivation of cells (Hela) from a case of cervical carcinoma. The cells were grown in a composite medium of chicken plasma, bovine embryo extract and human placental cord serum. Since then many lines have been developed and grown in relatively simple media. Eagle (1955) was able to serially cultivate these cells for months in chemically defined medium of amino acids, vitamins and salts, supplemented by 10% human serum. During the same year, Henle and Deinhardt (1955) used this cell line in primary isolation of mumps viruses and reported the superiority of this host system over inoculation of chick embryo. This was later confirmed by Utz et.al. (1957). Buckley (1959) was able to propagate arboviruses in Hela cells.

This cell line has been used for cultivation of many viruses and it is routinely used by many laboratories for virus studies.

Toolan (1954) reported the establishment of cells from a human epidermoid carcinoma (metastatic epidermoid carcinoma, primary in larynx) in tissue culture, which he called H.Ep2.

This cell line like Hela has found extensive application.

(Ginsberg, (1962) reported that adenoviruses of human origin produced a characteristic cytopathic effect consisting of rounding and aggregation of affected cells into clusters. This was observed more clearly when these agents were propagated on Hela and H.Ep2 cell lines; the CPE of these agents was slow in a number of primary cultures.

(v) Cells of Simian Origin: Primary cultures of monkey kidney cells have been used in virus studies more often than any other cultures because of their sensitivity to a large number of viruses. Their use however is not without problems and possible hazards. The use of this primary culture allows for possible presence of adventitious agents derived from the host which in some cases can infect the laboratory worker and also interfere with the outcome of virus growth. (Enders and Peebles, 1954; Rustigian et.al., 1955; and Anderson and Doane, 1972). The development of cell lines from virus-free monkey kidney tissues was therefore investigated and the success in this field has made available cell lines with a wide range of virus susceptibility.

Hull et.al. (1956) reported the establishment of LLC-MK₁ and LLC-MK₂ derived from rhesus monkey kidneys. The cells were grown on Earle's BSS with 20% filtered chick embryo extract and 30% horse serum and later adapted to medium 199 containing 1% horse serum. Preservation of the cell line was possible by freezing in either liquid nitrogen or dry ice. Hull et.al. (1962) reported that the cells could support the growth of a wide variety of viruses; adenoviruses and influenza did not however grow on these cell lines.

Hopps et.al. (1962) reported serial cultivation of kidney cells of African green monkey (AGMK) which they designated as BS-C-1. Cultures frozen and stored for months were successfully revived and propagated. Eleventh passage cultures were as sensitive to SV₄₀ as primary cultures and titration of polioviruses and measles virus in higher passage levels yielded similar end points compared to primary cultures. The cells were refractory to SV₂ but supported the multiplication of SV₁, 4 and 5 which did not grow in primary cultures. Veronelli et.al. (1962) reported the use of BS-C-1 in isolation of rubella virus from patients and remarked that this cell line could be substituted successfully for primary AGMK cultures in virus isolation work.

Yasumura and Kawakita (1963) isolated a new cell line, Vero, from AGMK cells and reported its wide range of susceptibility for arboviruses. Rhim and Schell (1967) demonstrated the usefulness

of Vero cells in rubella studies. They showed that whereas rubella was detectable in primary AGMK cells only by the interference method, this virus produced CPE easily at high dilutions in Vero cells. In their experience, a significant difference in virus titre was observed with different passage levels, eg. passage 112 was found to give CPE with higher dilutions earlier than passage 168 and also the end point titre of the former was at least one logarithm higher. Liebhaber, et.al. (1967) also reported the Vero cell cultures were slightly more sensitive than primary AGMK in rubella virus studies. Specimens were inoculated into Vero cells and pAGMK, for primary isolation. Out of a total of 25 specimens, 14 were positive in Vero whilst only 3 were positive on pAGMK and in no case was a specimen positive on pAGMK and negative on Vero cells. Plaques could also be formed easily on Vero cells by rubella virus.

(vi) Cells of Rodents: Primary rabbit kidney cell cultures have been found useful for cultivation of some viruses particularly those of the herpes group. Serial cultivation of rabbit kidney cells was investigated in order to make these cells readily available. Westwood, et.al. (1957) reported on the serial cultivation of tissues of different origins amongst which was embryo rabbit kidney, designated ERK1. This cell line was found to be sensitive to polioviruses. Beale, et.al. (1963) reported the establishment of a cell line RK13, from rabbit kidney cells which were susceptible

to the herpes virus group, vaccinia and simian adenoviruses. During the same year, McCarthy, et.al. (1963) reported on the use of this cell line in the primary isolation of rubella viruses. Distinct CPE was produced by the virus which could be seen as early as the 5th day. This was an improvement on the interference test with pAGMK which required nearly two weeks to complete. Hull et.al. (1965) used rabbit kidney cell line LLC-RK₁ developed in their laboratory for virus susceptibility tests. Virus spectrum was similar to that found with RK13. Rubella virus could also be propagated in this cell line which was refractory to polio 1, Cocksackie B5, A9, myxoviruses and the paramyxoviruses.

Kissling (1957) showed that primary hamster kidney cells were susceptible to a wide range of arboviruses and CPE was very distinct. The sensitivity of this tissue culture was found to be equal to mouse inoculation with eastern equine and Venezuelan equine viruses and superior for Illheus and West Nile viruses. These findings were later on confirmed by Diercks and Hammon (1958) who speculated that this system might provide a source for the preparation of diagnostic antigens and vaccines.

Stoker and MacPherson (1964) described a cell line, BHK-21 derived from cultures of kidneys from 1-day old syrian hamsters. They listed a wide range of viruses which could be propagated on this cell line, from vaccinia, herpes viruses, myxoviruses, paramyxoviruses to many arboviruses; polioviruses and rhinoviruses

did not grow on this cell line. Vaheri et.al. (1965) indicated that rubella viruses could grow with specific CPE in BHK-21. Plaques were also readily obtainable and titres were comparable to those obtained by observation of CPE. Suspension cultures of this cell line could also be utilized for production of high titre antigens of rubella.

(B) Maintenance of Tissue Culture Cells at Reduced Temperature

Cell cultures in monolayers can be maintained in a usable condition for 2-3 weeks at 37°C. In many cases, it is not possible to utilize all the cultures produced at once, and it becomes desirable therefore to preserve them for use at a future time. This may be accomplished by lowering incubation temperatures to reduce the metabolic activity of the cells.

Swim and Parker (1955) reported the storage of cell lines at 4°C without replacement of the medium. The cell lines studied included HeLa, MB13 isolated originally from mouse lymphosarcoma, CM2, a mixture of skin and muscles of chick embryo and FS4 and U12 from human foreskin and uterus respectively. They were able to maintain these monolayers for periods of 6 to 9 weeks. A large proportion of the cells contracted and became spherical within 2 or 3 days at 4°C. When they were withdrawn from storage and placed at 37°C without replacing the medium, the cells gradually assumed their original characteristics.

Weinstein et.al. (1956) maintained monolayer tubes of human primary amnion cells at 28°C. The medium was changed every 15 - 20 days and in this way cultures could be maintained without apparent damage for periods exceeding 6 weeks. The medium was again replaced when

these cultures were needed for virus studies. Control cultures lasted for 15 or more days before significant degeneration occurred. No studies were done to determine any change in susceptibility of the cells as a result of maintenance at reduced temperature.

Crawford (1958) described the storage of monkey kidney tissue cultures at 5°C for 5 - 6 weeks. He observed that the cells pulled apart, rounded up and left numerous intercellular spaces. These cells however spread to full sheet within 24 hours after re-incubation at 35°C. Susceptibility of stored cells to polioviruses showed that comparative titres could be achieved on refrigerated and non-refrigerated cultures. The authors reported that attempts to store HeLa, KB and monkey heart cells gave inconsistent results whilst rabbit kidney cells would survive at least 2 weeks of cold storage.

Schmidt (1969) reported that human amnion and monkey kidney cell cultures have been successfully maintained at room temperature for 4 to 5 weeks in their laboratory. Cells were incubated at 36° to 37°C for 1 day. Maintenance medium was then replaced before use for virus propagation.

Mahdy and Bansen (1970) demonstrated that young HEp-2 cultures were superior to cultures aged at room temperature in supporting the replication of Sindbis virus and echovirus type 11. Also aged RK-13 cell cultures were found much more resistant to vesicular stomatitis virus than young cultures. This resistance was attributed to production of interferon by the cells.

Doane and Anderson (1971) reported that Vero cells could be kept at room temperature for several days before and after inoculation and these could support the growth of virus from small inocula. Inoculated cultures were placed at 37°C for 2 - 3 days to stimulate virus replication and were examined by light and electron microscopy for CPE and virus particles respectively. Both cultures left at room temperature and those maintained at 37°C have comparable sensitivity to adenoviruses, Coxsackie B5 and influenza A2/Hong Kong viruses.

(C) Stability of Viruses

Virus studies by isolation on clinical specimens depend on infectious viruses in these specimens. Since viruses are affected by physical and chemical means, knowledge of the stability of these is important for proper handling of specimens. This portion of the review therefore gives a brief summary on the stability of viruses to environmental conditions.

(i) Picornaviruses

Robinson (1950) studied the effect of heat and pH on strains of coxsackie A, B and polioviruses. She reported that infectivity was completely lost when these viruses were held at 53°C for 30 minutes in 10% beef infusion broth. Loss of infectivity was more rapid when the viruses were suspended in distilled water. All strains survived at room temperature for 1 day in beef broth at pH 2 to 9. One strain however lost considerable activity at pH 2 in 1 day and all activity in 7 days.

Melnick and Ledinko (1950) working on stability of coxsackie viruses reported that at 25°C, the viruses were better protected when infected mouse brain was suspended in 50% serum than in 50% glycerol. However at 2°C, 50% glycerol gave better protection. There was a fall in titre of between 1.0 to 2.1 log. units at 25°C and less than 2 log. units at 2°C when tissue was stored for 6 days. Coxsackie viruses in human stool specimens underwent thermal inactivation at 55°C for 15 minutes but survived when suspended in milk. (Kaplan and Melnick, 1954)

Wallis and Melnick (1962) reported that high concentrations of Mg⁺⁺ and Ca⁺⁺ stabilized polio, echo and coxsackie viruses to heat treatment at 50°C for 1 hour. At 37°C the stability lasted for at least 3 days. The converse happened when these viruses were tested in the presence of sodium ions.

The stability of echoviruses at 4°C and higher temperatures varied for different types and strains (Melnick, 1965).

Rhinoviruses are acid labile in contrast to enteroviruses hence they are inactivated in fluids with pH between 3 and 5. These viruses in respiratory secretions or in tissue culture fluid were inactivated rapidly at 56°C and slowly at 4°C (Andrewes and Tyrrell, 1965).

(ii) Influenza and Parainfluenza viruses

Wallis and Melnick (1962) showed that influenza and parainfluenza viruses were more sensitive to heat in the presence of

Mg⁺⁺ and Ca⁺⁺ than when suspended in distilled water or saline solution. Treatment of parainfluenza viruses with 20% ether for 18 hours at 4°C resulted in complete loss of infectivity of all four types (Chanock et.al. 1963). The viruses were also rapidly inactivated at temperatures between 20°C and 37°C. The rate of inactivation was markedly influenced by the composition of the suspending medium.

Specimens collected in media containing protein eg. bovine albumin or gelatin and adjusted to pH 7.2 could be kept at 4°C for a few hours without loss in titre (Canchola et.al. 1965). For successful isolation of these viruses, specimens should be inoculated directly into tissue cultures without prior freezing and thawing.

Robinson and Dowdle (1969) reported that influenza viruses could be stored at -60°C without any loss in infectivity. However storage at ordinary freezing temperatures and at 4°C in the absence of added protein would result in loss of infectivity.

(iii) Other RNA Viruses

Beem et.al. (1960) reported that it was frequently impossible to reisolate respiratory syncytial virus (RSV) from nose and throat specimens that had been frozen at -30°C. Temperature stability studies with prepared suspensions showed that fluids with initial infectivity titres of 10⁵TCID₅₀/ml showed almost complete loss of infectivity after slow freezing at -30°C. Better preservation was achieved with rapid freezing at -70°C. Infectivity of RSV in tissue culture

during storage under various conditions of temperature and pH were investigated (Hambling, 1964). Results indicated that 90% infectivity was lost at 55°C after 5 minutes and about 1% of infectious virus remained after 7 days at 4°C. Infectivity at 4°C was maintained best at pH 7.5.

Reoviruses in maintenance medium placed at different temperatures for 14 days gave the following stability results:- decrease of 1.0 log. units at 4°C; 2.2 log. units at 24°C and 5.6 log. units at 37°C. The viruses were completely inactivated in 45 minutes at 56°C (Rhim et.al. 1961).

Rubella virus is relatively heat labile. Thermal inactivation is influenced by the nature of the suspending medium. Purified virus in the presence of 2% chicken serum or 1% bovine serum at 37°C was inactivated at the rate of 0.3 to 0.4 log. ID₅₀/hr. and showed complete loss of infectivity after 1 hour at 56°C (Parkman et.al. 1964). Higher protein content reduced thermal lability. Fabiyi et.al. (1966) reported that rubella viruses in tissue culture fluid were completely rendered noninfectious at 37°C and partially at 4°C, when tubes were held at these temperatures for 7 days.

In general, arboviruses are unstable and readily inactivated by relatively little heat, particularly on the acid side of neutrality than on the alkaline side (Hammon and Sather, 1969). These viruses are stable at -70°C. Infected tissues can be stored for months in neutral glycerol at 5°C.

(iv) Adenoviruses

Ginsberg (1956) investigated the stability of adenoviruses to temperature and pH alterations. At -70°C the viruses retained their titres for 19 months; 7 months at -30°C and 70 days at 4°C . At room temperature these viruses retained their infectivity for 10 to 14 days and were relatively stable at 22°C between pH 6.0 and 10.0. Adenoviruses were however less stable at acid pH.

Adenoviruses were stabilized at 50°C by Na^{+} whilst Mg^{++} enhanced their inactivation. (Wallis et.al. 1962) At room temperature no virus was detected in 1M. Mg^{++} after 7 days. Leibovitz (1969) reported that adenoviruses survived better in Amies transport medium containing agar than in throat washings. For isolation from throat washings or anal swabs, best results were usually obtained when unfrozen specimens were inoculated into tissue cultures within 6 hrs (Rose, 1969).

(v) Herpes Viruses

The effect of pH and temperature on herpes simplex virus has been studied by many workers. Farnham and Newton (1959) reported that loss of infectivity of herpes was very high on either side of neutral pH and that the virus was more labile on the acid side. The half life of herpes infectivity at 30° , 37° and 44°C was found to be 13, 3 and 0.4 hrs. This was later confirmed by Scott et. al. (1961).

Munk and Ackermann (1953) studied the stability of herpes sim-

plex virus at different pH levels of phosphate buffered saline at a constant temperature of 4°C. There was a loss of 0.2 log. unit increment as the pH was changed from 5.9, 7.1 and 8.0 within a 12 hour period. Mg⁺⁺ and Ca⁺⁺ enhanced thermal inactivation of herpes viruses and this was so rapid that even at 4°C the viruses were completely inactivated within 1 hr. (Wallis and Melnick, 1965). Na⁺ stabilized the virus and glycerine was found to be a better stabilizer.

(vi) Poxviruses

Poxviruses are more stable to environmental conditions than most of the other viruses. Wallis and Melnick (1962) however reported that smallpoxvirus was destroyed rapidly in the presence of divalent cations in molar concentrations. Hamparian et.al. (1963) also showed that at pH 3.0, poxviruses lost about 3.0 log. unit titre in 30 minutes when compared with stability at pH 7.2. The viruses can resist drying and dessication. Smallpox viruses could be recovered from crusts kept at room temperature for many months. In fluid suspensions, these viruses were destroyed by heating at 60°C for 10 minutes but dried vaccine withstood 100°C for 10 minutes (Downie, 1965; Downie and Kempe, 1969).

SUMMARY

Primary cell cultures are generally more sensitive than cell strains or cell lines for the isolation of most viruses from clinical specimens. Certain viruses are however isolated more easily on cell strains or cell lines than on primary cell cultures e.g. Hela cell cultures were very sensitive for the

isolation of mumps and respiratory syncytial viruses (Chanock and Parrott, 1965; Henle and Deinhardt, 1955) and rubella virus isolation rates were higher when BS-C-1 or Vero cell cultures were used (Rhim and Schell, 1967; Veronelli, et.al., 1962). Human fetal diploid cell cultures were reported to be more sensitive than primary rhesus monkey kidney cells for the isolation of rhinoviruses (Schmidt, et.al., 1965).

Herrmann (1967) reported that the use of serum free medium for cell cultures was more suitable for isolation of influenza viruses since some batches of serum seemed to inactivate the viruses.

The choice of cell type and media for isolation work therefore depend on the viruses expected in the specimens. It has been found that the parallel use of two or three cell culture systems, ideally primary monkey kidney cells, human diploid cell strain and a cell line contributes a higher number of viral isolates than in a single cell culture system.

II. SEROLOGY

Serologic procedures form the bulk of the work done in many virus diagnostic laboratories. These tests are done to confirm the patient's infection by demonstrating a rise in antibody against his own or the prototype virus strain. They are also performed to establish a diagnosis when no agent has been isolated, by screening acute and convalescent blood specimens for antibody rises against several viruses considered to be the likely aetiological agents. The most commonly used techniques are the 1) Complement fixation test (CF); 2) Haemagglutination inhibition test (HI) and 3) Neutralization test (NT).

(i) Complement Fixation Test: This method finds the widest application because of its simplicity, specificity and also because results can be obtained rapidly. Group specific CF antigens have been demonstrated for myxoviruses (Henle et.al., 1958) adenoviruses (Rowe et.al., 1957) and there is crossreaction among members of certain arbovirus groups (Casals, 1957, 1963). The use of these antigens allows diagnosis of infection with a virus in a particular group to be made without resort to the use of the many individual types within the group.

Complement fixing antibodies appear late in most infections and generally may fall to undetectable levels very rapidly during convalescence (Schmidt and Lennette, 1965). In rubella infections, CF antibody response rarely appeared before the second week of convalescence and failed to develop entirely in some patients (Parkman et.al. 1969). Hence the knowledge of the

status of this type of antibody response during infection determines whether CF test should be used.

The CF test is also used for studies on virus-host cell relationships, antigenic relationships between viral antigens and variants and the identification of viral isolates (Kwapinski, 1965).

Some infections in which CF can be used for diagnosis are: influenza A & B, parainfluenza 1, 2, 3, adenoviruses, herpes simplex virus, respiratory syncytial virus, mumps and varicella-zoster viruses.

(ii) Haemagglutination Inhibition Test: Many viruses have been shown to have the capacity to agglutinate the erythrocytes of certain mammalian or avian species. Specific antibody inhibits this agglutination and this reaction can therefore be used for antibody assay or virus identification. With some viruses the haemagglutinating factors are associated intimately with the varion eg. influenza while in others they are readily separable from the infectious unit eg. poxviruses (Hirst, 1965).

Haemagglutination by influenza viruses was the first to be described in 1941 by Hirst and since then this phenomenon has been described for poxviruses (Nagler, 1942) certain enteroviruses (Goldfield et.al. 1957) arboviruses (Sabin and Buescher, 1950; and Clarke and Casals, 1958) and for adenoviruses (Heubner et.al. 1958). In 1957 Stewart et.al. demonstrated haemagglutination for rubella viruses and reported that the factor responsible was in-

timately connected with the virion.

Haemagglutination-inhibition (HI) antibodies generally appear shortly after infection in advance of CF antibody. They persist for a longer time after infection. Specificity of these antibodies varies with different viruses. It is highly strain specific for influenza viruses (Henle et.al. 1958) whilst it gives broad spectrum of group reactivity for the arboviruses (Casals, 1949). The HI test for rubella was found to supply the full range of serologic information including the epidemiologic study of immunity patterns of a population (Parkman et.al. 1969). It can be used for typing isolates of adenoviruses, certain enteroviruses and reoviruses (Kwapinski 1965). It is not however useful with human sera for diagnosis of echoviruses due to heterotypic reactivity. Other virus infections in which HI test can be used for diagnosis are the parainfluenza viruses, mumps and Newcastle disease virus.

(iii) Neutralization Test: The high degree of immunologic specificity of this test makes it the standard against which other serologic methods are usually evaluated (Kwapinski, 1965). The virus neutralization test was first demonstrated in animal experiments by Sternberg in 1892 in a study of vaccinia (Carpenter 1965). Bodian (1949) described the neutralization test for polio virus type 1 strains using monkeys. Opton et.al. (1955) reported the use of this test for polioviruses in tissue cultures whilst Weller and Witton (1958) reported results for varicella. Since then the neutraliza-

tion test has been used for all other viruses that could be propagated in tissue cultures. Neutralizing antibodies generally appear very early during infection, usually ahead of HI and CF antibodies and persists longer than these two other antibodies (Schmidt and Lennette, 1965). Because of this long persistence, this test can be used in epidemiological studies.

III. ELECTRON MICROSCOPY IN DIAGNOSTIC VIROLOGY

Nagler and Rake (1948) pioneered the use of electron microscopy (EM) in diagnostic virology. Specimens from cases of variola, vaccinia and varicella were shadowed with gold and examined under the EM. They were able to identify these viruses on the basis of their gross morphology and size.

Van Rooyen and Scott (1948) also reported the use of the EM in smallpox diagnosis and were able to identify the virus from 4 out of 5 cases. Strauss et.al. (1949) demonstrated virus-like bodies in specimens obtained from skin papillomas. Brick-shaped elementary bodies similar to those seen in control molluscum contagiosum lesions were observed from specimens prepared by touching the lesions to the grid. Specimens from wart lesions were also diagnosed this way and the infections were confirmed by histological studies.

In 1959 Brenner and Horne described a negative staining method for electron microscopy of viruses using phosphotungstic acid (PTA) and this extremely simple method has contributed immensely to direct examination of specimens with the electron microscope. Almeida et.al. (1962) demonstrated varicella virus particles in specimens using this method whilst Smith and Melnick (1962) were able to identify herpes viruses from vesicular lesions. Doane et.al. (1967) reported the identification of parainfluenza and mumps viruses from nasopharyngeal secretions and cerebrospinal fluid respectively.

Joncas et.al. (1969) also reported on successful application of the EM in direct examination of infected materials. They were able to identify respiratory syncytial and parainfluenza viruses and this extended the catalogue of viruses that could be identified directly from specimens.

Doane et.al. (1969) reviewed the applications of EM in virus diagnosis and suggested that viruses isolated in cell cultures could be

identified readily. Their report (Anderson and Doane, 1970) re-emphasized the applications and the limits of electron microscopy in diagnostic virology and observed that rapid diagnosis could be made on specimens within hours as compared to days by isolation techniques. Anderson and Doane (1972) reported that adventitious viruses in cell cultures could be detected by the EM and in this way the use of contaminated cultures could be avoided.

The use of electron microscopy has been taken a step further. Almeida and Waterson (1969) reviewed and discussed the usefulness of visualizing virus-antibody interaction under the EM. This method referred to as immune electron microscopy has been used extensively in serum hepatitis studies. Chaudhary et.al. (1971) also described serologic cross-reaction in the enterovirus group by immune EM which could facilitate rapid identification of these viruses.

EXPERIMENTAL WORK

PART ONE

COMPARISON OF VIRUS DIAGNOSTIC RATES IN A ROUTINE LABORATORY AND SPECIAL INVESTIGATION AT THE FAMILY PRACTICE UNIT OTTAWA CIVIC HOSPITAL

INTRODUCTION

Viruses are known to be very susceptible to environmental conditions (Melnick and Ledinko, 1950; Beem et.al., 1960 and Robinson and Dowdle, 1969) as a result of which specimens collected from patients may become non-infectious during transportation. To minimize this effect and allow for optimal virus isolation from specimens, modifications of transport media used in bacteriology had been tested using viruses (Leibovitz, 1969). The results indicated that some of the constituents inactivated some viruses eg. thioglycollate in Amies medium inactivated herpes simplex, coxsackie and myxoviruses. Also certain ingredients like charcoal and agar needed to be of high quality since some batches could be toxic to tissue culture cells. Wallis et.al. (1962) described the inactivating effect of Ca^{++} and Mg^{++} on vaccinia, herpes and adenoviruses and also the effect of Na^+ on enteroviruses. These ions usually find their way into solutions used for specimen collection.

Holzel et.al. (1963) reported that virus isolation rates could be increased when specimens were inoculated directly into tissue cultures. Another report in 1965 from the Manchester Public Health Laboratories showed that isolation rates were again higher and earlier when direct inoculation procedures were used. Herrmann (1967) described a system where specimens obtained from hospital patients were inoculated into cultures within 2 hours

after collection. He reported that virus isolation rates were much higher when compared to results from routine laboratories.

This portion of the thesis re-emphasizes the fact that direct inoculation of specimens into cultures should be the aim of virus diagnostic service. Cell cultures were supplied to the Family Practice Unit (FPU), a satellite clinic of the Ottawa Civic Hospital, for immediate inoculation with specimens. Comparison was made between virus diagnostic rates of the routine laboratory and the FPU investigations, both of which used the same facilities. The effect of storage of specimens at 4°C overnight on re-isolation of virus was also studied.

MATERIALS AND METHODS

TISSUE CULTURES

Primary African green monkey Kidney (pAGMK):

These were purchased as trypsinized suspensions from Connaught Medical Research Laboratory, Toronto, Ontario, Canada, and later on from Grand Island Biological Co., New York. Cultures were made in tubes with 1.5 ml of $1 - 2 \times 10^5$ cells/ml in growth medium consisting of Earle's MEM with non essential amino acids, 0.2% w/v glucose and 10% fetal calf serum (FCS). Antibiotics were added in the following concentrations:- 100 iu/ml penicillin; 100 μ g/ml streptomycin; 1 μ g/ml Fungizone and 1% anti PLO (Gibco). The tubes were incubated at 37°C in stationary racks for 5 - 7 days during which time confluence was reached. Growth medium was changed to maintenance medium made up of Earle's minimum essential medium (MEM) with non-essential amino acids, 0.2% w/v galactose and 2% FCS. Antibiotics concentrations were the same as in growth medium.

Human Fetal Lung Cells (HFL):

Procedures used for preparation of these cells were essentially the same as described by Hayflick and Moorhead (1961). Human fetuses from therapeutic abortions were received and the lungs were dissected out. The lung tissues were cut into pieces and then washed with phosphate buffered saline (PBS). These were then digested with trypsin and the cells were spun down. Cells were resuspended in growth medium and seeded into bottles and incubated at 37°C. When monolayers were formed, the cells were split 3 in 1 and grown to monolayers again. Cells at low passage levels were stored

frozen in ampoules at -70°C in growth medium containing 10% glycerol and 5% diethylglycol. Ampoules were retrieved when needed and the cells seeded and serially passaged. Cells were usually used at passage level 13 or 14 by putting up cultures in tubes. Growth medium and maintenance medium were similar to those used for pAGMK.

Human Fetal Kidney Cells (HFK):

Kidneys from fetuses were decapsulated and then processed as in the case for the lung tissues. When the cells reached confluence, they were either stored frozen or passaged once and then seeded into tubes. In this case the cells were used at 2nd passage level. Growth and maintenance media were similar to those described for pAGMK.

Specimens

Throat washing/swabs, anal swabs and or stool were obtained for virus isolation. Acute and convalescent sera taken 21 days apart were used for serology. Blood from infants was obtained by finger puncture method. 0.2 ml of blood taken with lambda pipette was put into a tube containing 0.6 ml veronal buffer. This was centrifuged and the supernatant fluid taken as 1:8 dilution of serum assuming a haematocrit of 50%.

Virus Isolation

0.1 ml of throat washing and of saline extract of rectal swab (1-2 ml of saline per swab) or of 10% saline suspension of stool were inoculated into at least one of each type of tissue culture. Stool suspension and extracts were first clarified by centrifugation. Tubes were incubated at 37°C in stationary racks and examined every day for CPE. Hemadsorption was

performed on the lung cells on the 5th and 14th day before cultures were discarded. The number of HFL cultures inoculated per specimen was increased to four tubes when HAd viruses were found to have increased in the community or during an anticipated influenza outbreak. Viruses were then identified by the HAd reversal test (HRT) or CPE.

Virus Identification Procedures

(i) Hemagglutination Inhibition (HI) Test: Medium from the cultures were tested for hemagglutination (HA) and when positive, the agent was identified using the following antisera:- influenza A2 and B, parainfluenza 1, 2, 3; mumps and SV5. These sera were purchased from Microbiological Associates, Maryland, USA and treated to remove non-specific inhibitors as recommended by WHO Committee (1959). The micro HI technique was used; 0.025 ml of virus suspension containing 4 HA units and 0.025 ml of serum dilutions were incubated at room temperature for 1 hour. 0.025 ml of 0.75% guinea pig rbc suspension was added to each well and incubated for 30 minutes. Results were read by observing the serum which inhibited HA at the highest dilutions.

(ii) Hemadsorption Reversal Test (HRT): This method was developed by Phipps (1969) and as the name implies, it involves reversal of hemadsorption (HAd) by homologous serum. Human fetal lung cell cultures inoculated with throat washing/swab were tested for HAd agents with guinea pig red blood cells (rbc). A drop of 10% rbc

suspension was added to the cell culture without removing the medium. Cultures which showed positive HAd were washed with PBS and 1.5 ml of predetermined dilution of antiserum treated to remove non specific inhibitors added. 1.5 ml of PBS was added to one positive culture to serve as control. Cultures were held at room temperature for 20 minutes and observed for reversal of HAd by the light microscope.

The dilutions of the antisera used for the test were determined by titration for HI titres, against homologous and heterologous antigens. The highest dilution which gave specific inhibition with homologous antigen was chosen as the working dilution.

Antisera used were goat immune lactoglobulin against influenza A2/Hong Kong (supplied by Dr. C. Mitchell, University of Ottawa) influenza A2/Japan and influenza B antisera from commercial sources.

(iii) Neutralization Test: Neutralization tests in tissue culture tubes were done as described by Schmidt and Lennette (1965). Equal volume of the virus diluted to contain 100 TCID₅₀/0.1 ml and anti-serum (1:20) were incubated at room temperature for 1 hour and the mixture inoculated into tissue cultures. Identification of enteroviruses was done by the "intersection serum scheme" of Schmidt et. al. (1961) which involved antisera pool. Inhibition of HAd was used in identification of myxo- and paramyxoviruses which could not be typed by the HI or HRT method. This was done where the virus was other than influenza A or B or when HA titre was too low for HI test.

Complement Fixation Test

The micro CF method described by Sever (1962) and modified by Mingle (1969) was used. 3 units of complement, 3 minimal haemolytic doses (MHD) of hemolysin, 1 optimal dilution of antigen and 2% sheep rbc suspension were used. Complement fixation was done overnight at 4°C. Haemolytic system was then added and incubated at 37°C for 30 minutes. 0.025 ml of reagents were used. Antigens used for screening sera were:- influenza A soluble, influenza B soluble, parainfluenza 1, 2, 3, adenovirus, respiratory syncytial virus, herpes simplex, mumps soluble and viral; Mycoplasma hominis and pneumoniae.

EXPERIMENTAL PROCEDURES

Effect of Storage on Virus Titre

(i) Direct Comparison Using Natural Specimens: Throat washings were obtained from patients attending the Family Practice Unit (FPU), Ottawa Civic Hospital and inoculated immediately into tissue cultures. The cultures were placed in an incubator at 37°C. The specimens were stored at 4°C overnight at the FPU. The inoculated cultures and the specimens were transported to the routine laboratory on the following day where duplicate cultures were inoculated using the stored specimens. The same batches of cultures were used. All cultures were examined daily by the light microscope for CPE.

(ii) Prepared Specimens: Suspensions of prototype viruses maintained in our laboratory were made and titrated using pooled throat washings as diluent. 0.1 ml of the suspensions was inoculated into 4 tubes of pAGMK per dilution. These dilutions were held overnight at 4°C and duplicate cultures were re-inoculated using the stored specimens. The cells were examined by the light microscope.

(iii) Indirect Comparison: Virus isolation rates and serology of the FPU investigations and the routine laboratory were compared.

RESULTS

Effect of Holding Specimens at 4°C

(i) Direct Comparison: Highest virus isolation rate is likely to be achieved if specimens could be inoculated into tissue culture immediately after collection (Holzel, 1963). Since many of the specimens received in the routine laboratory are held overnight at 4°C, it is important to determine this effect on the outcome of virus isolation from clinical specimens. Specimens were therefore inoculated into tissue cultures immediately and after storage and the results compared. 256 specimens were tested in this way. 0.1 ml of each specimen was inoculated into at least one tube of each of the following tissue cultures:- pAGMK, HFL and at times into HFK when available. Mostly throat washings were used since these were collected in quantities which would permit inoculation of many tubes. Table 1 shows the results when re-isolation was attempted with positive influenza virus specimens. All five specimens were negative after these had been stored at 4°C overnight. When positive clinical herpes simplex specimens were also stored overnight, 5/6 gave positive re-isolation (Table 2). CPE was however delayed when the refrigerated specimens were inoculated. These results clearly indicate that there was loss in infectivity when specimens were stored. The results represent those specimens in which valid comparison could be made since in some attempts the cells in one group degenerated or were contaminated and as such these cells were discarded before CPE could appear.

(ii) Prepared Specimens: The range of viruses obtained with the clinical specimens was narrow and therefore prepared specimens were

used. Throat washings were seeded with viruses and quantitatively assessed in the fresh state and again after overnight storage. Five viruses, namely influenza A2/Hong Kong, coxsackie B₄, adenovirus type 7 and echovirus type 9, were used. Each dilution of each virus was inoculated into 4 tubes of tissue culture and incubated at 37°C for 7 days. Hemadsorption test was used in determining the end point with influenza virus and CPE for the other viruses, and the results are presented in Table 3.

Influenza A2/Hong King virus showed an average loss of 0.7 log. units of infectivity as a result of storage overnight at 4°C. Robinson and Dowdle (1969) reported that storage of influenza at 4°C in the absence of added protein could affect infectivity. This may explain the loss of infectious virus in the specimens, since throat washings were obtained with saline solution without protein. The strain of coxsackie B₄ used in our experiment was found to be stable at least after storage at 4°C overnight; similar results were obtained with adenovirus type 7. Herpes virus concentration was reduced by a minimum of 0.9 log. units after storage. Munk and Ackermann (1953) reported a loss of 0.4 log. units at pH 7.1 over a 12 hour period when virus was suspended in PBS. The echovirus type 9 in throat washing showed 2 log. unit reduction in titre after storage.

(iii) Indirect Comparison: An indirect method of ascertaining the effect of holding specimens at 4°C was undertaken by comparing virus diagnostic rates in the routine laboratory with the Family Practice Unit investigation. Tables 4 and 5 show that comparison.

Serologic diagnostic rates between the two systems showed the same

range of viruses (Table 4). Higher diagnostic rates were however readily obtainable in the FPU investigations than in the routine laboratory. This may be due partly to the fact that people in the general population would not have easy access to the doctor and therefore not seen in early stage of illness. "Acute" blood specimen would therefore have high enough titre which would prevent demonstration of the usual four fold rise in titre with the "convalescent" specimen. Another reason was that all those who gave acute phase specimens were not available for convalescent specimens and in this way some positives could be missed. In the FPU most of the patients gave the second blood specimens for serology. Notwithstanding these reasons, serologic diagnostic rates in FPU were twice that in the routine laboratory in the first period and three times during the second period under review.

Comparison of virus isolation rates as shown in Table 5 once again gave higher rates with FPU. The results here are very interesting in that although the range of viruses in both groups were the same as shown by serology, isolations in the two systems were very different. During 1970, only one influenza virus was isolated in the routine laboratory whilst 7 isolates of influenza A and 2 influenza B were made in FPU investigations. Specimens sent to the routine laboratory had usually been kept over a period before being sent to the laboratory. Some infectious viruses might have been inactivated during storage and transportation. During the 1971 period, seven influenza B and 11 adenoviruses were isolated in the FPU and only 1 adenovirus isolated in the routine laboratory.

Table 6 shows the overall virus diagnostic rates as determined by

percentage of specimens with positive virus isolation or serology or both. The percentage was 33.3 in the FPU and 12% in the routine laboratory investigation.

Speed of Reporting Virus Isolation

The speed of reporting virus isolation is of importance to the attending physician. Such information may help in some instances to determine whether antibiotics should be prescribed or what immunization procedure to take as far as contacts are concerned or to determine the prognosis. Early reporting can also help the physician in developing an awareness of the virus and its clinical associations. Rates of reporting 47 virus isolates, 29 from the routine laboratory and 18 from the Family Practice Unit were compared. Herpes simplex isolates were excluded since the rapid development of characteristic CPE by this virus clouds the differences observed with other viruses. The results of that analysis of the data are set out in Tables 7 and 8.

6.8% of the results in the routine laboratory were telephoned to the doctors on the 3rd day whilst 44.4% were made in the FPU investigation. On the 7th day 61.1% of the virus isolates were reported to the doctors in the FPU and 51% in the routine laboratory. Fig. 1 shows the graphical representation of the comparison between the two systems. Significant differences were found between the routine laboratory and the FPU on days 3, 4 and 10. The percentage of isolates reported in the FPU on the 3rd day was very high and these isolates were reported at a time when the physician would be actively involved with treatment of the illness. This shows clearly

that results could be obtained earlier by the direct inoculation of specimens which was employed in the FPU. This point was also demonstrated with the direct comparison of the natural specimens.

Effect of Interval Between Onset of Disease and Collection of Specimens on Laboratory Diagnostic Rate

The earlier the specimen is taken the more likely it is that isolation will be successful. Similarly, the earlier the first (acute) specimen of serum is obtained, the more likely it is that a diagnostic four-fold rise in antibody titre will be demonstrated. The results of the specimens processed through the FPU over a two year period were analysed as shown in Tables 9 and 10. Higher virus isolation rates were obtained when specimens were collected within the first four days after onset of illness. For serology the period seemed to extend to 7 days. Figure 2 illustrated the results for both virus isolation and serology. It appears that the greatest success of diagnosis of virus infection may be anticipated between the first and the seventh day after onset of illness. The relatively low diagnostic rate during the early stage of illness is contrary to expectation and this may be due to uncertainty of clinical diagnosis during that stage, leading to poor selection of cases.

Significance of Number of Specimens Submitted Per Patient on Laboratory Diagnosis

The taking of specimens for laboratory examination has met with some degree of antipathy from patients. There is particular resistance to the taking of rectal swabs, stool specimens and convalescent blood samples especially when the patient has fully recovered.

An analysis was therefore made to determine the effect of the number

of specimens submitted per patient on the incidence of diagnostically positive results. Records of 1,332 patients in the routine laboratory for the year 1969 were analysed, counting two paired sera as a single specimen. The results of the analysis are shown in Table 11. It is clear from the results that the proportion of positive results increases in a logarithmic series. Percent positive results were 3.8% with 1 specimen, 8.3% with 2 specimens, 15.5% with 3 specimens and 30% with 4 specimens. Similar results were obtained when the specimens were analysed according to age groups (Table 12). This indicates that the increase in percent positives with each addition of specimen is real and independent of age group.

The number of specimens submitted per patient may also indicate the keenness of the clinician involved as well as the clinical diagnosis. It is reasonable to assume that where at least paired sera and another specimen were submitted to the laboratory, the clinical diagnosis was presumed to be viral and that during the intervening period between acute and convalescent phases no bacterial diagnosis was obtained. The submission of multiple specimens may indicate the strong conviction of the clinician that the disease was of viral origin and as such every relevant specimen should be obtained for successful laboratory diagnosis. Single acute blood samples only, or single throat swab or any other specimen could indicate that virology may have been done only as a formality. In some cases where only acute blood specimen was given, this could be due to patients not bothering to give convalescent sample after they had recovered or that bacterial diagnosis was made during the acute phase and as such the clinician did not order for the convalescent blood sample.

TABLE 1

COMPARISON BETWEEN IMMEDIATE INOCULATION OF SPECIMEN
AND STORAGE AT 4°C OVERNIGHT BEFORE INOCULATION

EXPERIENCE DURING INFLUENZA A2/HK OUTBREAK IN
DECEMBER 1969 - JANUARY 1970

PATIENT NO.		ISOLATION RESULTS
VP 39	(F)	Positive Hemadsorption on Day 1 in HFL
	(C)	Negative Hemadsorption on Day 1 and Day 7
VP 43	(F)	Positive Hemadsorption on Day 3
	(C)	Negative Hemadsorption on Days 3 and 7
VP 46	(F)	Positive Hemadsorption on Day 1
	(C)	Negative Hemadsorption on Days 1 and 7
VP 47	(F)	Positive Hemadsorption on Day 3
	(C)	Negative Hemadsorption on Days 3 and 7
VP 66	(F)	Positive Hemadsorption on Day 1
	(C)	Negative Hemadsorption on Days 2 and 7

(F) = Immediate inoculation of specimen (throat washing)

(C) = Storage at 4°C overnight before inoculation

TABLE 2

COMPARISON BETWEEN IMMEDIATE INOCULATION OF SPECIMEN
AND STORAGE AT 4°C OVERNIGHT BEFORE INOCULATION
(HERPES SIMPLEX VIRUS)

PATIENT NUMBER	VIRUS TYPE ISOLATED	CELL TYPE USED	PERIOD IN DAYS BEFORE CORRESPONDING DEGREE OF CPE FIRST APPEARED		
			1+	2+	3+
VP 78	(F) Herpes Simplex	pAGMK	2	3	4
	(C) " "	"	3	6	Not attained
VP 170	(F) Herpes Simplex	"	-	-	3
	(C) " "	"	3	4	7
VP 121	(F) Herpes Simplex	"	3	4	5
	(C) " "	"	7	10	Not attained
VP 181	(F) Herpes Simplex	"	-	3	4
	(C) No virus isolated	"	-	-	-
VP 228	(F) Herpes Simplex	"	2	-	3
	(C) " "	"	2	3	5
VP 314	(F) Herpes Simplex	"	3	-	6
	(C) " "	"	5	6	8

(F) = Immediate inoculation of specimen

(C) = Storage at 4°C overnight before inoculation

TABLE 3

EFFECT OF STORAGE AT 4°C OVERNIGHT ON INFECTIVITY
OF VIRUSES IN THROAT WASHINGS

VIRUS TYPE		CELL TYPE INOCULATED	DILUTION AT WHICH SPECIMENS WERE POSITIVE (HAD OR CPE)		
			EXPT. 1	2	3
Influenza A2/ Hong Kong	(F)	Human Fetal Lung	10 ^{-3.0}	10 ^{-2.9}	10 ^{-2.6}
	(C)	" " "	10 ^{-2.0}	10 ^{-2.3}	10 ^{-2.0}
Coxsackie B4	(F)	Primary AGMK	10 ^{-6.0}	10 ^{-5.3}	
	(C)	" "	10 ^{-6.0}	10 ^{-5.3}	
Herpes Simplex	(F)	Primary AGMK	10 ^{-3.3}	10 ^{-2.0}	10 ^{-3.1}
	(C)	" "	10 ^{-2.0}	10 ^{-2.0}	10 ^{-2.2}
Adenovirus Type 7	(F)	Primary AGMK and Human Fetal Kidney	10 ^{-4.0}	10 ^{-4.4}	
	(C)	" " "	10 ^{-4.0}	10 ^{-4.4}	
Echovirus Type 9	(F)	Primary AGMK	10 ^{-6.0}	10 ^{-6.0}	10 ^{-8.0}
	(C)	" "	10 ^{-4.0}	10 ^{-4.0}	10 ^{-6.0}

(F) = Immediate inoculation of specimen

(C) = Specimen kept at 4°C overnight before inoculation

TABLE 4

COMPARISON OF SEROLOGICAL DIAGNOSTIC* RATES OF
ROUTINE LABORATORY AND FAMILY PRACTICE UNIT INVESTIGATION

LABORATORY	METHOD OF DIAGNOSIS	PERIOD FROM - TO	NO. OF CASES	VIRUSES DIAGNOSED					TOTAL	POSITIVE DIAGNOSTIC RATE %
				Flu A	B	Para	Adeno	Others		
Routine Laboratory	CFT	Jan. 1970-Dec. 1970	390	26	1	2	3	27	59	15.1
		Jan. 1971-Sep. 1971	232	1	2	1	2	9	15	6.4
Family Practice Unit	CFT	Jan. 1970-Dec. 1970	158	17	10	7	1	14	49	30.9
		Jan. 1971-Sep. 1971	141	3	9	2	7	8	29	20.6

* = 4-fold or greater rise in antibody titres between acute and convalescent sera

TABLE 5

COMPARISON OF VIRUS ISOLATION RATES OF
ROUTINE LABORATORY AND FAMILY PRACTICE UNIT INVESTIGATION

LABORATORY	METHOD OF DIAGNOSIS	PERIOD FROM - TO	NO. OF CASES	VIRUS ISOLATED					TOTAL	VIRUS ISOLATION RATES %
				Flu A	B	Para	Adeno	Others		
Routine Laboratory	Virus Isolation	Jan. 1970-Dec/70	1040	1	-	3	3	47	54	5.1
		Jan. 1971-Oct./71	602	-	-	-	1	13	14	2.3
Family Practice Unit	Virus Isolation	Jan. 1970-Dec./70	185	7	2	3	-	7	19	10.2
		Jan. 1971-Oct./71	176	-	7	1	11	7	26	14.7

TABLE 6

COMPARISON OF VIRUS DIAGNOSTIC RATES OF
ROUTINE LABORATORY AND FAMILY PRACTICE UNIT INVESTIGATION

JANUARY - DECEMBER 1970

	PERCENTAGE POSITIVE		OVERALL* DIAGNOSTIC RATE
	BY VIRUS ISOLATION	BY SEROLOGY	
Routine Laboratory	5.1%	15.1%	12%
Family Practice Unit	10.2%	30.9%	33.3%

* = Diagnostic rate indicates positive virus isolation
or serology or both

TABLE 7

DAYS AFTER RECEIPT OF SPECIMENS BEFORE VIRUS ISOLATION WAS REPORTED
BY ROUTINE LABORATORY (JANUARY - DECEMBER 1970)

VIRUS TYPE	DAYS AFTER RECEIPT OF SPECIMENS BEFORE ISOLATION WAS MADE												TOTAL			
	1	2	3	4	5	6	7	8	9	10	11	12		12+		
Influenza A	1*															1
Enterovirus		1		1		1		3		2	1	5				14
Parainfluenza				1				1			1					3
Adenovirus				1	1	1	1									4
Vaccinia				1	5											6
Hemorrhagic fever virus											1					1
TOTAL	1	1	0	4	6	1	2	0	4	0	2	3	5			29
Cumulative TOTAL	1	2	2	6	12	13	15	15	19	19	21	24	29			
Cumulative %	3	7	7	21	41	45	52	52	66	66	72	83	100			

* = Number of isolates

TABLE 8

DAYS AFTER RECEIPT OF SPECIMENS BEFORE VIRUS ISOLATION WAS REPORTED
BY FAMILY PRACTICE UNIT (JANUARY 1970 - JANUARY 1971)

VIRUS TYPE	DAY AFTER RECEIPT OF SPECIMEN BEFORE VIRUS ISOLATION WAS MADE												TOTAL		
	1	2	3	4	5	6	7	8	9	10	11	12		12+	
Influenza A	2*	1	4			1									8
Enterovirus			1												1
Parainfluenza						1	1								2
Adenovirus									1			1			1
Hepatitis A virus				1				1	4						6
TOTAL	2	1	5	1	0	0	2	1	1	4	0	1	0		18
Cumulative TOTAL	2	3	8	9	9	9	11	12	13	17	17	18	18		
Cumulative %	11	17	44	50	50	50	61	66	72	94	94	100	100		

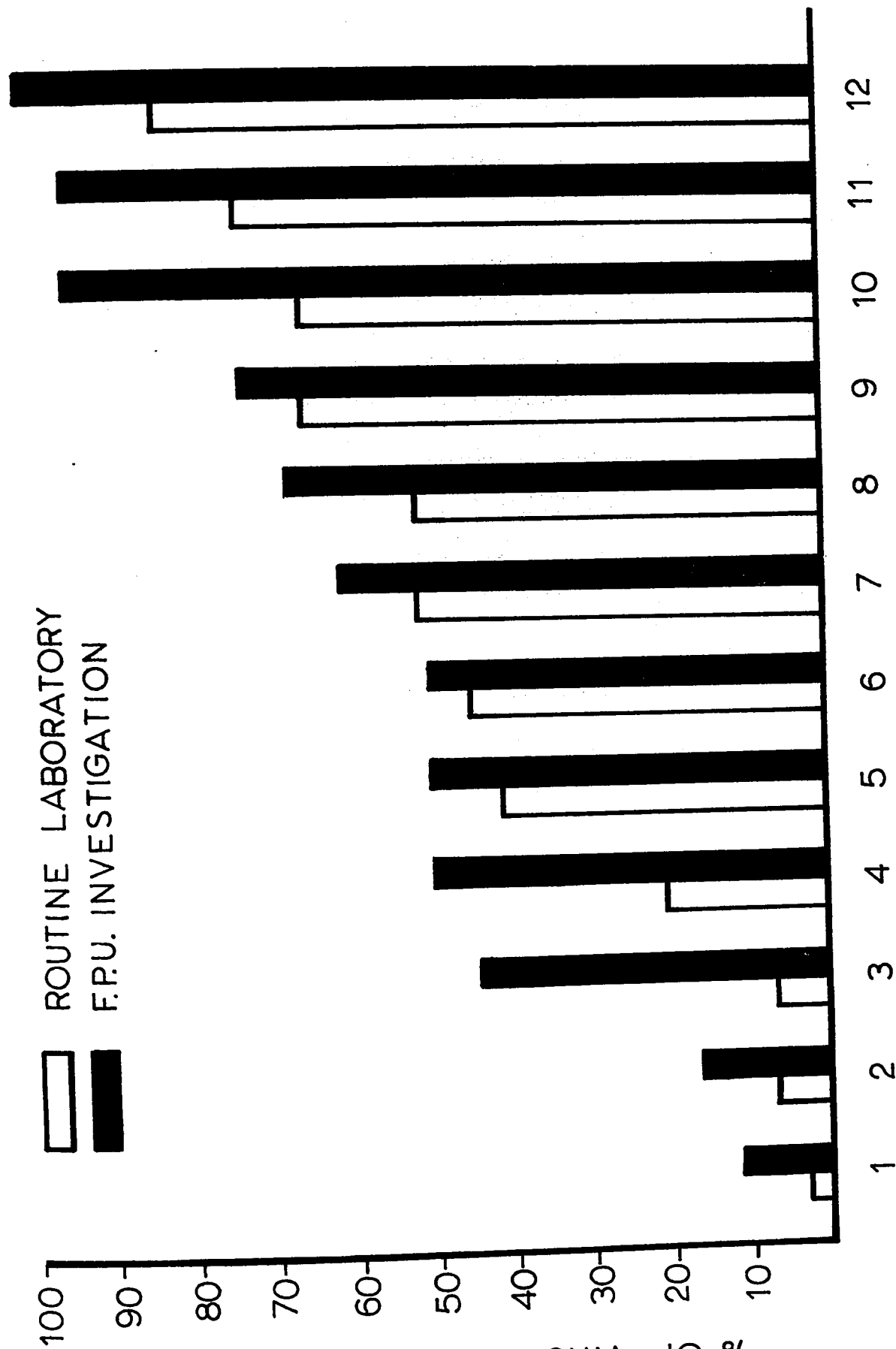
* = Number of isolates

Fig. 1

COMPARISON OF SPEED OF REPORTING VIRUS ISOLATES
IN ROUTINE LABORATORY AND FAMILY PRACTICE UNIT INVESTIGATION

% OF VIRUS ISOLATES REPORTED.

ROUTINE LABORATORY
F.P.U. INVESTIGATION



DAY AFTER INOCULATION OF SPECIMEN.

TABLE 9

*SEROLOGIC DIAGNOSTIC RATE:
EFFECT OF INTERVAL BETWEEN ONSET OF DISEASE
AND COLLECTION OF SPECIMENS (JANUARY 1970 - JANUARY 1972)

NUMBER OF DAYS BETWEEN ONSET OF DISEASE AND COLLECTION OF SPECIMENS	NUMBER OF SPECIMENS SHOWING		TOTAL	% POSITIVE (SEROLOGY)
	NEGATIVE SEROLOGY	POSITIVE SEROLOGY*		
Day 0	10	1	11	9
1	34	11	45	24
2	53	21	74	28
3	44	17	61	28
4	28	12	40	30
5	15	9	24	38
6	9	3	12	25
7	13	3	15	20
8	1	0	1	0
9	2	0	2	0
10	15	1	16	6

* = four fold rise in antibody titre between acute and convalescent sera

TABLE 10

VIRUS ISOLATION RATE:
EFFECT OF INTERVAL BETWEEN ONSET OF DISEASE
AND COLLECTION OF SPECIMENS (JANUARY 1970 - JANUARY 1972)

Day	NUMBER OF DAYS BETWEEN ONSET OF DISEASE AND COLLECTION OF SPECIMENS	NUMBER OF SPECIMENS SHOWING		TOTAL	% POSITIVE VIRUS ISOLATION
		NEGATIVE ISOLATION	POSITIVE ISOLATION		
0		10	1	11	9.0
1		39	6	45	13.3
2		63	11	74	14.8
3		47	14	61	22.9
4		34	6	40	15.0
5		22	2	24	8.3
6		11	1	12	8.3
7		14	1	15	6.6
8		1	0	1	0
9		2	0	2	0
10		15	1	16	6.2

Fig. 2

EFFECT OF THE DAY AFTER ONSET OF ILLNESS BEFORE SPECIMENS WERE COLLECTED
ON LABORATORY DIAGNOSTIC RATE IN FAMILY PRACTICE UNIT INVESTIGATION

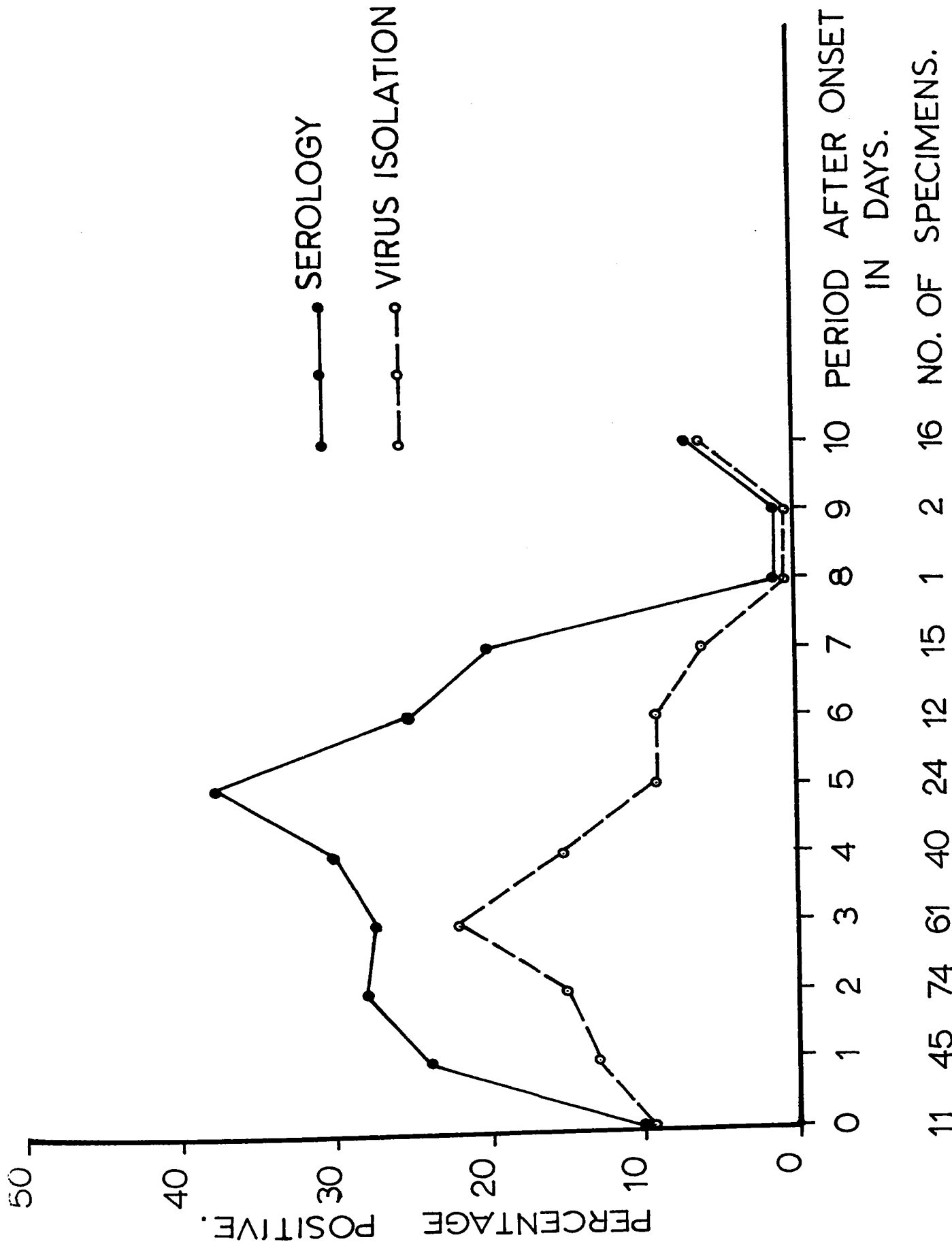


TABLE 11

VIRUS DIAGNOSTIC RATE:
EFFECT OF NUMBER OF DIFFERENT TYPES*
OF SPECIMENS SUBMITTED PER PATIENT

	NUMBER OF SPECIMEN TYPES/PATIENT				TOTAL
	1	2	3	4	
Total Patients	784	350	188	10	1,332
Positive results	30	29	29	3	91
Percent positive	3.8	8.3	15.5	30	7.5

* repeat specimens of the same type were not counted.

TABLE 12

DIAGNOSTIC RESULTS IN RELATION TO
NUMBER OF SPECIMEN TYPES SUBMITTED BY AGE OF PATIENT

AGE GROUP	NUMBER OF SPECIMENS RECEIVED											
	1			2			3			4		
	Pts.	+ve	%	Pts.	+ve	%	Pts.	+ve	%	Pts.	+ve	%
0-12	311	18	6	257	22	12	151	22	15	8	3	35
13-29	82	2	2.5	36	2	6	16	2	12.5	1	-	-
30-	268	7	2.8	40	4	10	18	5	30	-	-	-
NK	123	3	2.4	17	1	5.9	3	0	0	-	-	-
TOTAL	784	30	3.8	350	29	8.3	188	29	15.5	10	3	30

TOTAL: 1,332 Patients

+ve: Overall = 7%

Pts.: Patients

NK: Not Known

* repeat specimens of the same type were not counted

DISCUSSION

Stability studies on viruses have indicated that infectivity is lost or reduced when suspensions are maintained at 4°C. Beem et.al. (1960) reported that re-isolation of RSV from nose and throat washings that had been frozen at -30°C was impossible in most cases. Andrewes and Tyrrell (1965) reported that rhinoviruses in respiratory secretions or tissue culture fluid were inactivated at 4°C. Influenza viruses were also inactivated at 4°C when suspensions were stored at that temperature in the absence of added protein (Robinson and Dowdle, 1969). Our results of re-isolation experiments with the positive influenza specimens indicated that re-isolation attempts failed after storage of the 15 specimens at 4°C overnight. With the herpes simplex specimens, CPE was always delayed when refrigerated specimens were used. The loss in infectivity was demonstrated by the stability test with the prepared specimens. There was a loss of 2 log. units with echoviruses type 9 through storage overnight. Melnick (1965) reported that the stability of echoviruses at 4°C varied for different types and strains. This may explain the reduction in titre which we noticed.

When Table 1 is re-examined in the light of the results with influenza virus stability test as shown in Table 3 it seems that influenza virus concentrations in these specimens were low and also the strain of virus involved was very susceptible to environmental conditions. Our experience with egg inoculations also gave us the impression that the virus was readily adaptable to eggs since hemagglutination was easily demonstrated with allantoic fluids. This is in direct agreement with the observations made by Pereira et.al. (1969) and Satz et.al. (1970) that the Hong Kong variant has

been easy to isolate in eggs.

Virus isolation rates of 10.2% and 14.7% were obtained during the 1970 and 1971 periods respectively in the FPU investigations. Our rates were slightly better than the rate of 8.3% obtained in similar investigations in general practice in Manchester (1965). In another investigation in four hospitals, these workers obtained isolation rates ranging from 38% to 11%. Their upper limit was attributed to the frequency with which young cells were sent to the hospitals. The highest results were obtained when cells were replaced twice a week. In our case, cells were replaced once a week. The difference in our results may also be due to the cell types used. We used pAGMK, HFL and later on HFK cells whilst the Manchester group used pAGMK and Hela cells which were found to be more sensitive for primary isolation of adenoviruses and RSV. These viruses formed the bulk of viruses isolated by this group. Other reports had indicated the superiority of Hela cells over pAGMK cells in primary isolation of adenoviruses and RSV (Ginsberg, 1962; Chanock and Parrott, 1965).

It is therefore apparent that for maximum success with virus isolation, tissue culture cells supplied to satellite clinics for immediate inoculation should be replaced as often as possible. Also cell types of various origins should be used. Ideally primary monkey kidney cells, a diploid cell strain and a continuous cell line should be used to cover most of the viruses which may be encountered in the general population.

Our analysis of the results on speed of reporting virus isolation to the doctors, indicate that better diagnostic aid could be provided by the

FPU investigation method. Herrmann (1967) reported that using direct inoculation procedures 67.6% of all virus isolates were reported within 7 days after receiving the specimens. In the FPU investigation 61.1% of isolates were reported within the same period as against 51.7% reported by the routine laboratory. Comparison of the diagnostic rates together with the high percentage of isolates reported by the 3rd day in the FPU indicate that not only could higher isolation rates be obtained by the FPU but that a higher percentage could be reported at a time when treatment of disease was still active. A method of immediate inoculation of specimens could provide a diagnostic service compatible with medical practice.

Fig. 2 illustrates the frustrations which one may face in virus diagnosis if specimens are not taken during the early acute phases of illness. Within the first 4 days, isolation rate was 10% or more and this dropped afterwards. The likelihood of recovering virus is therefore greatly diminished after 4 days. Serologic diagnostic rates also fell sharply with specimens which were first taken after the 7th day of illness.

The results on the significance of number of specimens submitted per patient indicated that higher diagnostic results could be achieved when the maximum number of relevant specimens were obtained per patient. These specimens should always include paired blood samples for serology since more diagnosis was possible by serology.

The next part of the thesis outlines how immediate inoculation of specimens into tissue cultures could be achieved.

PART TWO

MAINTENANCE OF PRIMARY AFRICAN GREEN MONKEY KIDNEY (pAGMK) AND VERO CELLS AT ROOM TEMPERATURE (25°C). A SYSTEM FOR VIRUS ISOLATION IN COMMUNITY PRACTICE

INTRODUCTION

We have shown in part one of the thesis that storage at 4°C could deprive most specimens of infectious virus particles. Immediate inoculation of specimens into tissue cultures had also been shown to provide better diagnostic rates and early results. One question which has to be resolved if such a system could be used widely is the supply of tissue cultures to hospitals and doctors' offices for direct inoculation. Normal storage of tissue culture at 37°C may entail extra expenditure. An alternative would be to store cell cultures at room temperature ready for inoculation. Weinstein et. al (1956) successfully stored human amnion cell cultures at 28°C without apparent damage to the viability and ability of these cells to support polioviruses. Mahdy and Bansen (1970) however reported that susceptibility of HEp-2 cell cultures to infection with Sindbis and echo 11 viruses was altered after these cells had been maintained at room temperature.

This part of the thesis reports on studies done to determine the effect of maintenance at room temperature on the sensitivity of pAGMK and Vero cell cultures. This was done to assess whether such a procedure could be used in providing tissue cultures to hospitals and satellite clinics for immediate inoculation with specimens. These cells were also tested for their susceptibility to prototype viruses. The possible uses of such a maintenance procedure in field studies are discussed.

MATERIALS AND METHODS

TISSUE CULTURES

pAGMK:

This was purchased as trypsinized suspension from Connaught Medical Research Laboratories, Toronto, Ontario, Canada and later on from Grand Island Biological Co., New York. The handling of these cells has been described in part one (materials and methods).

Vero Cells

These were kindly supplied by Professor Frances Doane of the School of Hygiene, University of Toronto, as a monolayer in a 4 oz. prescription bottle. Cells were kept at 37°C for 2 days to reactivate growth and then trypsinized. These were serially grown in the same growth medium used for pAGMK and stored frozen in ampoules at -70°C. Ampoules were retrieved and the cells passed once in bottles before they were seeded into culture tubes (1.5 ml of 0.6×10^5 cells/ml). These were then handled in the same way as the pAGMK.

Antibiotics concentrations in both media were as follows:
100 iu/ml penicillin, 100 µg/ml streptomycin, 1 µg/ml fungizone and 1% GIBCO APPLO.

Maintenance of Cells at Room Temperature (RT): Batches of the cells in tubes which had reached confluence were kept at 37°C. Groups of these were removed each day for four successive days and placed at room temperature (25°C). On the 5th day, a group which had been kept entirely at 37°C (control) together with the cells maintained at room temperature were inoculated with viruses.

Sensitivity of Cultures Maintained at RT: Predetermined dilutions of viruses in phosphate buffered saline (PBS) were inoculated simultaneously into the group of cells. At least 4 tubes were inoculated for each group and each virus. Cells were kept at 37°C and examined daily for CPE by the light microscope. The degree of CPE and day of its first appearance were noted.

Viruses used were coxsackievirus types B4 and B5, echovirus type 9, vaccinia, herpes simplex and influenza A2/Hong Kong.

Virus Yield of Cells: Cell cultures maintained at room temperature and control cells were inoculated with 100 TCID₅₀/0.1 ml of coxsackievirus type B4 and incubated at 37°C. The supernatant fluids were pooled within groups after control cells showed 4+ CPE and titrated. Virus yields were calculated by Kärber's method and expressed as log. TCID₅₀/1.0 ml.

Cell Susceptibility to Viruses: Prototype viruses maintained in tissue cultures in our laboratory were titrated in parallel in pAGMK and Vero cells which had been held exclusively at 37°C. Most of the viruses had been passaged in pAGMK. The source of these viruses are indicated by the suffixes. Some of the viruses were passaged 3 times in Vero cells and then retitrated on both cell types. 0.1 ml of 10 fold dilutions were inoculated into 2-4 tubes per dilution. The day when a particular dilution first produced definite CPE and the highest dilutions showing CPE after 7 days of incubation were noted. In the case of adenoviruses incubation period was 10 days. Virus titres were calculated by Kärber's method.

Virus Isolation from Clinical Stool Specimens: 8 clinical stool

specimens from which enteroviruses had been isolated were kindly supplied by the Virus Laboratory, Central Public Health Laboratory, Toronto, Ontario, Canada. Suspensions were made and clarified. 0.1 ml of the suspensions were inoculated into 3 tubes each of pAGMK and Vero cells and incubated at 37°C.

Comparison between immediate inoculation of viruses into cells maintained at RT and Cells inoculated with refrigerated specimens:

pAGMK cells which had been maintained at RT were inoculated with virus suspensions in throat washings and held at room temperature for 3 days before incubation at 37°C. These same suspensions were held at 4°C for 3 days and inoculated into the same batch of cells which had been kept entirely at 37°C. Number of days post incubation at 37°C, before CPE first appeared was noted.

Electron Microscopy:

Cell lysates from pAGMK cultures which had been held at room temperature before and after inoculation were examined by electron microscopy for virus particles. The cells were scraped after 48 hours incubation at 37°C and pelleted. The pellets were resuspended in distilled water and subjected to a series of freezing and thawing to release virus particles. Grids were prepared according to the procedures reported by Doane et.al. (1969). A drop of the cell lysate was placed on a large drop of distilled water resting on a parafilm. Formvar-carbon coated grid (400 mesh) was touched quickly to the surface of the drop and a drop of 2% sodium phosphotungstate (PTA) with about 0.1% bovine serum albumin at pH 7 was placed on the grid. The PTA was allowed to stain the specimen for 30 - 60 sec.

and the excess fluid was removed with filter paper. The grid was then placed in plastic petri dish and allowed to dry in air. This was then examined using Philips EM300 at a magnification of 18,000-45,000 with a double condenser illumination and acceleration voltage of 60KV. 35mm camera with Kodak films was used for photography.

RESULTS

Morphology of Cells Maintained at Room Temperature (RT):

Series of experiments were done to determine what changes may take place in the morphology of cells after they had been held at room temperature. At least six tissue culture tubes of pAGMK and Vero cells which had reached confluence were removed from the 37°C incubator each day for 4 successive days and held at room temperature. On the 5th day, six other culture tubes which had been held entirely at 37°C were removed and these together with tubes held at room temperature were examined under the light microscope.

Cells which had been held at RT curled up and left intercellular spaces. Rounded cells were also found scattered throughout the monolayer and more of these were seen in tubes which had been held at RT for 4 days. Figure 3 shows these typical morphological changes as seen with pAGMK. Tissue culture cells which had been held at RT however assumed the normal appearance after they had been re-incubated at 37°C overnight.

Cell Sensitivity:

Since cells held at RT are to be used for virus isolation, it is desirable to know the effect on their sensitivity when they are so treated. In a series of experiments pAGMK and Vero cells were held at RT for 1 to 4 days as previously described. On the 5th day a final sample of tubes was removed and all the tubes were simultaneously inoculated with viruses and then incubated at 37°C. 4 tubes of each cell type held at RT for the specific period were used for each virus.

The effect on the sensitivity of pAGMK cultures maintained at RT is shown in Table 13. Of the five viruses tested, only coxsackievirus type B4, and herpes simplex were affected; echo 9 and vaccinia did not seem to be affected. Storage of human lung cells at room temperature for 1 day did not affect the sensitivity to influenza virus. The results indicate that the pAGMK cells left at room temperature for 4 days became less sensitive to infection with herpes simplex and coxsackievirus type B4.

CPE first appeared in the control cells with herpes simplex on day 2 and was complete by the 4th day. But cells kept at room temperature for 4 days first showed CPE after 5 days incubation and this was complete on the 7th day. CPE was also delayed in cells maintained at room temperature for 4 days when these were inoculated with coxsackievirus type B4.

Six viruses were also used to test the sensitivity of Vero cells held at RT. The results of these experiments are shown in Table 14. It will be seen that the Vero cells did not show a clearcut loss in sensitivity to any of the viruses used.

Quantitative assessment of cell sensitivity was also done. pAGMK and Vero cells were again held at room temperature as previously described. These were inoculated with 100 TCID₅₀/0.1 ml of coxsackievirus type B4 and incubated at 37°C. This virus was chosen since the results of the qualitative test on pAGMK showed that the cells held at RT were less sensitive to this virus.

Supernatant fluids from the inoculated cultures were pooled within groups after the cells held entirely at 37°C before inoculation had shown

4+ CPE. These were titrated for virus yields and the titres were calculated by Karber's method. The results are shown in Tables 15 and 16. The loss in sensitivity of pAGMK held at room temperature was indicated by the lower titres obtained from these cells. Again the Vero cells did not seem to be affected. The results give quantitative confirmation of the conclusions derived from the observation of rate of development of CPE in Tables 13 and 14.

Cell Susceptibility to Viruses:

Susceptibility of pAGMK and Vero cells held at 37°C to prototype viruses maintained on pAGMK were compared to determine their relative sensitivities. 19 viruses representing both RNA and DNA viruses were titrated in parallel in the two types of cells. 0.1 ml of 10⁻¹ dilutions were inoculated in 2-4 tubes per dilution. Virus titres were calculated by Karber's method and the sensitivities compared. Table 17 shows the results. Distinct CPE was produced by coxsackievirus type A9 in pAGMK cells. Dilutions of 10⁻⁷ produced CPE within 2 days after inoculation. This virus failed to produce CPE in Vero cells at the initial dilution of 10⁻¹.

The coxsackie B viruses readily caused CPE in both pAGMK and Vero cells at lower dilutions and CPE was extensive. However, at higher dilutions CPE was only extensive in pAGMK while little islands of degenerated cells were visible in Vero cells. It took relatively shorter time for end points to be reached in pAGMK and with the exception of coxsackievirus type B1, titres were at least 1 log. unit higher on pAGMK than on Vero cells.

Marked variation in infectivity titres was obtained when echoviruses were titrated. Three out of the four viruses used, had been passaged only on fetal lung cells hence variation was not due to adaptation of the viruses to pAGMK.

Adenoviruses also gave reasonable differences when titrated on both cells. The CPE at low dilutions in Vero cells was found to be more extensive.

Vero cells showed slightly higher susceptibility to herpes simplex viruses than pAGMK; equal sensitivity to vaccinia; sensitivity to reovirus type 1 and polio viruses was lower on Vero cells.

Echovirus type 9, coxsackievirus type B4 and adenovirus type 7 gave higher titres on pAGMK with pAGMK passaged viruses and these differences were maintained even after passage in Vero cells; with poliovirus type 3, the difference in titre was reversed.

Fig. 4, illustrates some of the differences in susceptibility of the cells to the viruses. This shows clearly that although in some cases end point titres were reached at the same time and sometimes later in pAGMK (see also Table 17), these cells were more sensitive than Vero cells.

Virus Isolation from Clinical Specimens:

pAGMK and Vero cells were again compared for sensitivity to viruses using 8 clinical stool specimens. 0.1 ml of clarified suspensions of specimens from which enteroviruses had been isolated were inoculated into 3 tubes of each cell type and incubated at 37°C. Table 18 shows the results of that experiment. Coxsackievirus type A9 was isolated in pAGMK but not in Vero cells. Maintenance medium from Vero cell cultures inoculated with the cox-

sackievirus type A9 positive specimens was blind passaged into pAGMK. No CPE developed after 7 days incubation which showed that the Vero cells were indeed not susceptible to coxsackie A9 virus. The coxsackie B4 viruses were isolated on both pAGMK and Vero cells but CPE usually appeared first on pAGMK. Echovirus type 9 was also isolated on both cells.

Comparison Between Immediate Inoculation of Viruses into Cells Held at RT (aged) and Inoculation of Refrigerated Specimens into Fresh Cultures:

If cells held at room temperature were to be recommended for immediate inoculation, then this system should be compared to simulated cases which might exist in the routine laboratory. In a series of experiments throat washings were therefore seeded with six viruses and inoculated in the fresh state into pAGMK cells which had been held at room temperature for 3 and 4 days respectively. The seeded throat washings were then stored at 4°C for 3 days and inoculated into the same batch of cells which had been held entirely at 37°C. The cultures were incubated at 37°C and examined daily for first appearance of definite CPE. The results in Table 19 indicate that in all cases, the aged cells showed early CPE. With coxsackieviruses type B4, herpes simplex and echovirus type 9 (100 TCID₅₀), CPE appeared in 2 days (after placing at 37°C), in the aged cultures inoculated immediately with specimens as compared with fresh cultures inoculated with refrigerated specimens which required 4 days at 37°C to show definite CPE. There was a marked difference also with coxsackievirus : type A9 and 10 TCID₅₀ echovirus type 9. No CPE was observed after 7 days of incubation when refrigerated specimen of echovirus type 9 was inoculated whilst immediate inoculation of specimen prior to refrigeration produced CPE in 2 days.

With influenza A2 in human fetal lung cell cultures, hemadsorption was detected after 6 hours incubation at 37°C in cells inoculated immediately and then held at RT overnight.

Electron Microscopy:

Early experiments showed changes in morphology of cells held at RT. Examination of such cells inoculated with specimens for viral CPE is likely to be misleading at times. Cell lysates of cultures held before and after inoculation were therefore examined for virus particles by the negative staining technique with PTA.

Fig. 5 shows the results. Virus particles were easily seen. Both full and empty virus particles were seen and the preincubation at reduced temperature did not seem to affect virus replication. In fact, reduced temperatures had been used by many workers to produce attenuated mutants of poliovirus, arboviruses, measles and influenza A virus for vaccine production (Van Kirk et.al., 1971).

Fig. 3

MORPHOLOGY OF pAGMK CELLS MAINTAINED AT ROOM TEMPERATURE

- A. Cells maintained entirely at 37°C
- B Cells maintained at room temperature for 3 days
- C. Cells maintained at room temperature for 4 days
- D. Cells maintained at room temperature for 4 days
and incubated at 37°C overnight

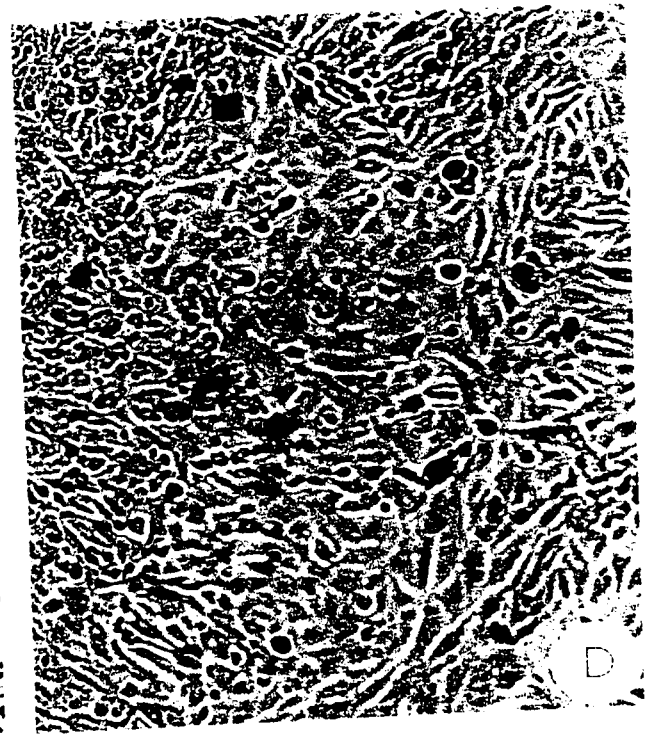
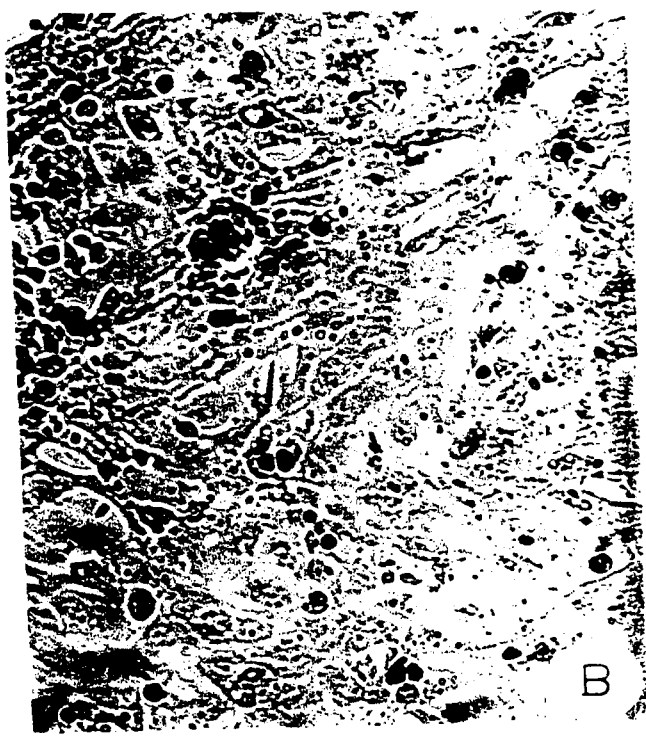
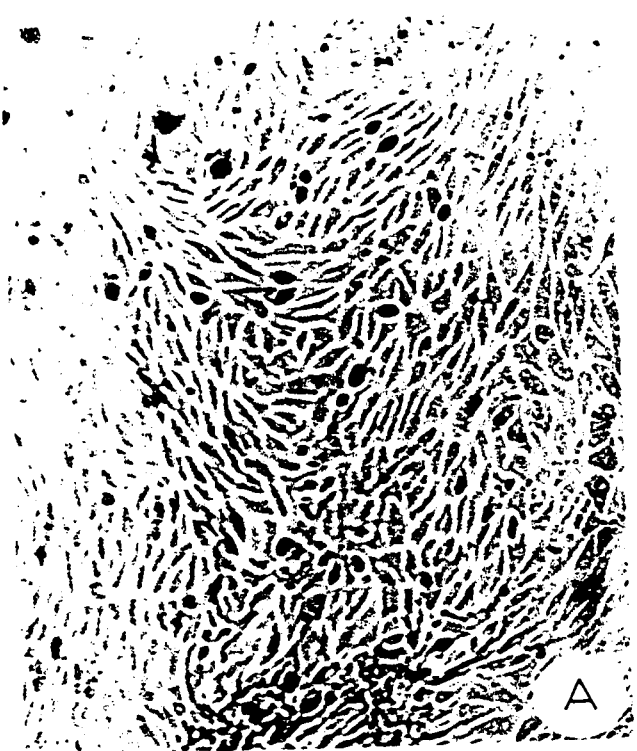


TABLE 13

EXPERIMENT ON SENSITIVITIES OF pAGMK CELL CULTURES MAINTAINED AT 37°C
AND ROOM TEMPERATURE RESPECTIVELY

Virus type inoculated	Degree of CPE or H.A.D. observed on corresponding day	Cultures held entirely at 37°C (Control)	Cultures maintained at Room Temperature for			
			1 day	2 days	3 days	4 days
Echovirus type 9	+	1 ^a	1	1	1	1
	++	-	-	-	-	-
	+++	2	2	2	2	2
Herpes simplex	+	2	3	4	4	5
	++	3	4	-	-	7
	+++	4	5	5	5	-
Vaccinia	+	1	1	2	2	ND
	++	2	2	-	-	
	+++	3	3	3	3	
Coxsackie B4	+	1	-	2	2	3
	++	-	2	-	3	4
	+++	2	3	3	4	5
Influenza A2/Hong Kong	+	1	-	ND	ND	ND
	++	2	2			
	+++	3	3			

a Days before corresponding degree of CPE appeared
 + Few foci of CPE
 ++ Moderate foci of CPE
 +++ Extensive foci of CPE
 ND Not done
 - Not attained

TABLE 14

EXPERIMENTS ON SENSITIVITIES OF VERO CELLS MAINTAINED AT 37°C
AND ROOM TEMPERATURE RESPECTIVELY

Virus type inoculated	Degree of CPE observed on corresponding day	Cultures held exclusively at 37°C (fresh)	Cultures maintained at Room temperature for			
			1 day	2 days	3 days	4 days
Coxsackie B4	+	3*	3	3	3	5
	++	4	4	4	4	
	+++	6	6	6	6	6
Coxsackie B5	+	3	3	3	3	3
	++	4	4	4	4	
	+++	6	6	5	6	4
Echo type 9	+	5	5	5	5	5
	++	7	7	7	7	7
	+++	-	-	-	-	-
Vaccinia	+	-	-	-	-	-
	++	3	3	3	3	3
	+++	4	4	4	4	4
Herpes simplex	+	1	1	1	1	
	++	2	2	2	2	2
	+++	3	3	3	3	3
Adeno type 7	+	3	4	3	3	3
	++	4	5	4	4	4
	+++	6	6	6	6	6

* Number of days before corresponding CPE was observed.

+ Few foci of CPE

++ Moderate CPE

+++ Extensive CPE

- Not attained

TABLE 15

EFFECT OF MAINTENANCE OF pAGMK CELLS AT ROOM TEMPERATURE
ON YIELD OF COXSACKIE B4 VIRUS

Number of days pAGMK were held at room temperature	Virus yield in supernatant fluid 48 hours after inoculation (\log_{10} TCID ₅₀ /ml)			
	Expt. 1	2	3	Average
0	7.75	7.5	7.25	7.5
1	6.5	6.5	6.5	6.5
2	6.25	6.5	6.75	6.5
3	6.0	6.0	6.0	6.0
4	4.0	4.25	3.75	4.0

TABLE 16

EFFECT OF MAINTENANCE OF VERO CELLS AT ROOM TEMPERATURE
ON YIELD OF COXSACKIE B₄ VIRUS

Number of days Vero cells were held at room temperature	Virus yield in supernatant fluid 4 days after inoculation (\log_{10} TCID ₅₀ /ml)		
	Expt. 1	2	Average
0	6.5	6.5	6.5
1	7.5	7.5	7.5
2	7.5	7.5	7.5
3	7.0	7.5	7.25
4	6.5	7.0	6.75

TABLE 17

COMPARISON BETWEEN SUSCEPTIBILITY OF PRIMARY AFRICAN GREEN MONKEY
(pAGMK) AND VERO TISSUE CULTURE CELLS TO DIFFERENT VIRUSES

VIRUS TYPE	pAGMK		VERO	
	D	T	D	T
Coxsackie				
A9(G)*	2	7.5	7	<1.0
B1(G)	2	6.0	3	6.5
B2(G)	3	7.5	5	6.0
B3(G)	3	7.5	3	4.5
B4(G)	4	7.0	5	6.0
B5(G)	3	7.0	3	6.0
B6(G)	3	6.5	7	5.0
Echoviruses				
6(L)	6	6.5	4	4.0
9(G)	2	>8.5	4	5.5
11(L)	5	5.5	7	4.0
30(L)	7	5.0	3	3.0
Adenoviruses				
4(K)	5	3.5	6	1.5
7(K)	3	3.5	3	1.5
Herpes simplex				
(V)	4	5.0	5	5.5
(K)	5	3.0	4	4.0
Vaccinia				
(G)	5	5.0	3	5.0
Polioviruses				
1(BSC)	4	6.75	3	6.25
2(G)	4	5.0	3	3.0
3(G)	3	5.5	4	5.0
Reoviruses				
1(G)	5	5.5	5	3.5
Echo 9 (V)	5	8.0	4	4.5
Coxsackie B4(V)	3	8.0	7	7.0
Polio 3(V)	5	6.5	4	7.0
Adeno 7(V)	3	3.5	6	2.5

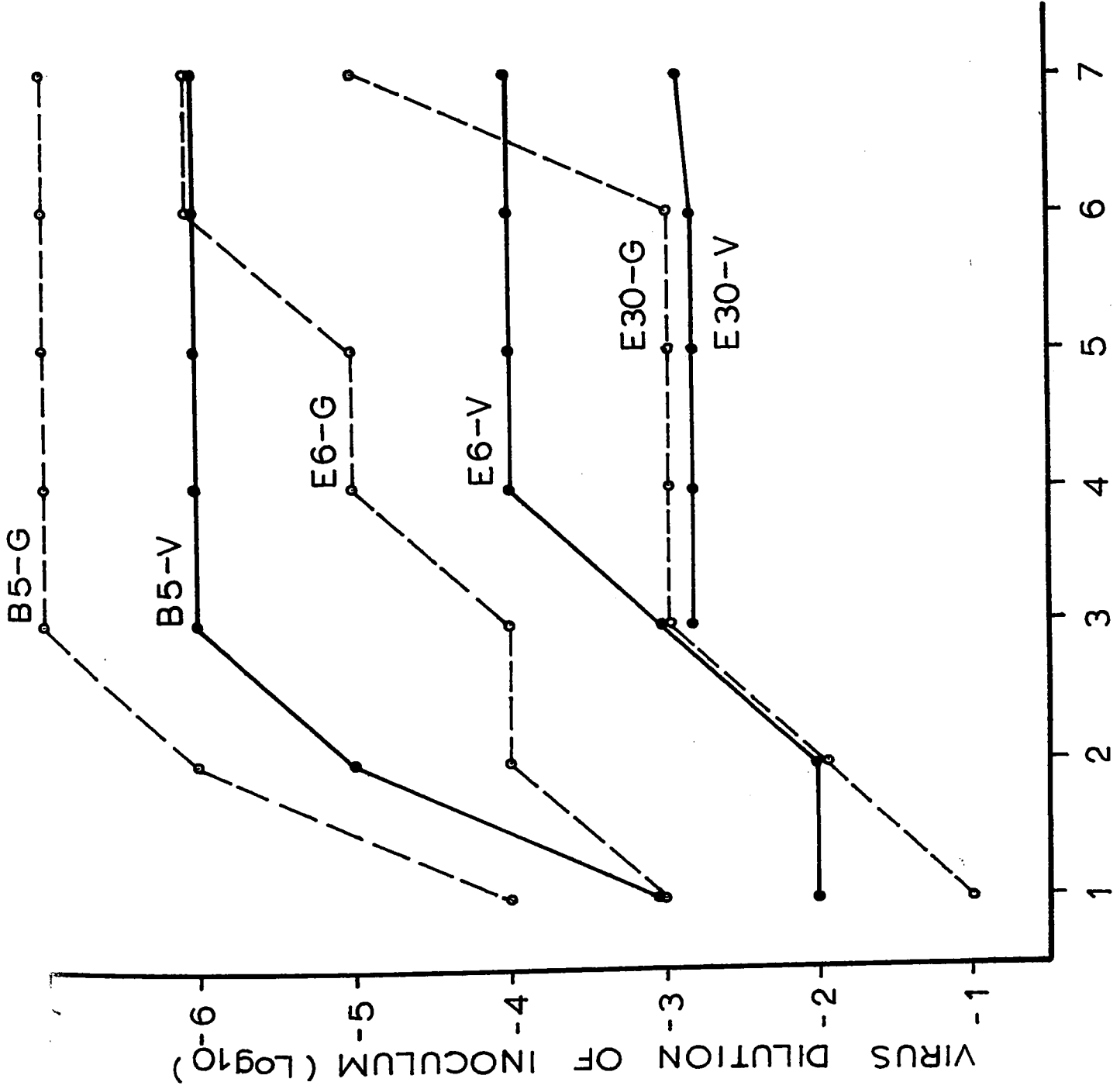
BSC = BS-C-1
 L = Human fetal lung cells
 D = Days before maximum titre was
 obtained
 * = Source of virus

G = pAGMK
 K = Human fetal kidney cells
 V = Vero cells
 T = Virus titre in Log TCID₅₀/0.1 ml.

Fig. 4

COMPARISON OF SUSCEPTIBILITY OF pAGMK AND VERO CELLS
MAINTAINED EXCLUSIVELY AT 37°C TO VIRUSES MAINTAINED ON pAGMK

N.B. B5 = Coxsackievirus type B4, E6 = Echovirus type 6,
E30 = Echovirus type 30, "G" and "V" after virus
type indicate pAGMK and Vero cells respectively on
which viruses were titrated.



DAYS AFTER INOCULATION WHEN DEFINITE CPE APPEARED.

TABLE 18

ISOLATION OF ENTEROVIRUSES
FROM CLINICAL STOOL SPECIMENS

Virus Type	Number isolated on:	
	pAGMK	Vero
Coxsackie A9	1	0
Coxsackie B4	6	6
Echo 9	1	1

TABLE 19

COMPARISON BETWEEN IMMEDIATE INOCULATION OF VIRUSES INTO "AGED" CELL CULTURES
AND INOCULATION OF REFRIGERATED SPECIMENS INTO "FRESH" CULTURES

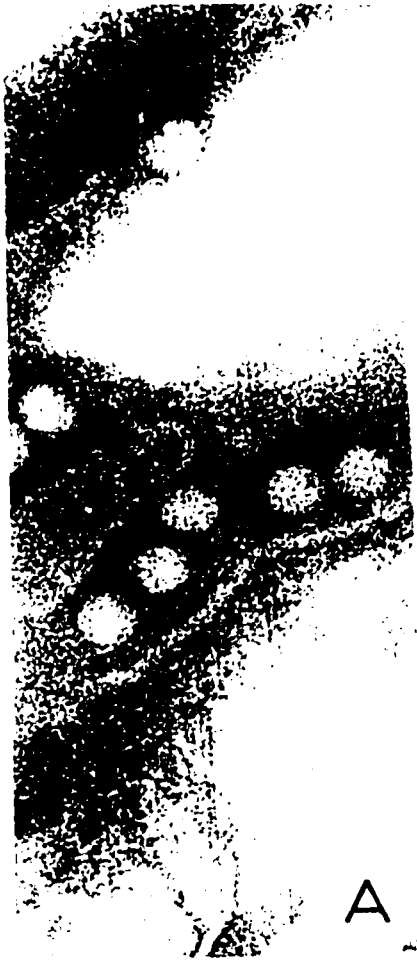
Virus type	Inoculum dose TCID ₅₀ /0.1 ml	Number of days specimens held at 4°C	Number of days pAGMK cells held at RT		Days (at 37°C) before defi- nite CPE first appeared
			Inoculation Pre	Post	
Coxsackie B4	10	0	4	3	2
	10	0	3	3	2
	10	3	0	0	4
Coxsackie A9	100	0	4	3	1
	100	0	3	3	1
	100	3	0	0	5
Echo 9	100	0	4	3	2
	100	0	3	3	2
	100	3	0	0	4
	10	0	4	3	2
	10	0	3	3	2
	10	3	0	0	Negative after 7 days
	100	0	4	3	2
Herpes simplex	100	0	3	3	2
	100	3	0	0	4
	100	0	4	3	1
Vaccinia	100	0	3	3	1
	100	3	0	0	2
	100	0	0	1	6 hrs.*
Influenza* HK/68	100	1	0	0	>6 hrs.*
	30	0	0	1	+ve overnight* 6 hrs.
	30	1	0	0	>6 hrs.+ve* overnight
	30	0	0	1	6 hrs.

* Human diploid fetal lung cells (HFL) were used instead of GMK.
Hemadsorption test was done with guinea pig cells instead of CPE.

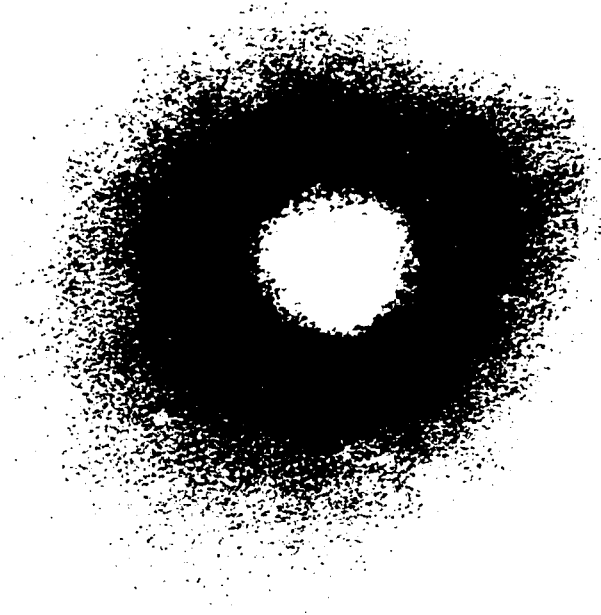
Fig. 5.

ELECTRON MICROGRAPHS OF VIRUSES FROM TISSUE CULTURE CELL LYSATES
NEGATIVELY STAINED WITH PHOSPHOTUNGSTIC ACID (PTA)

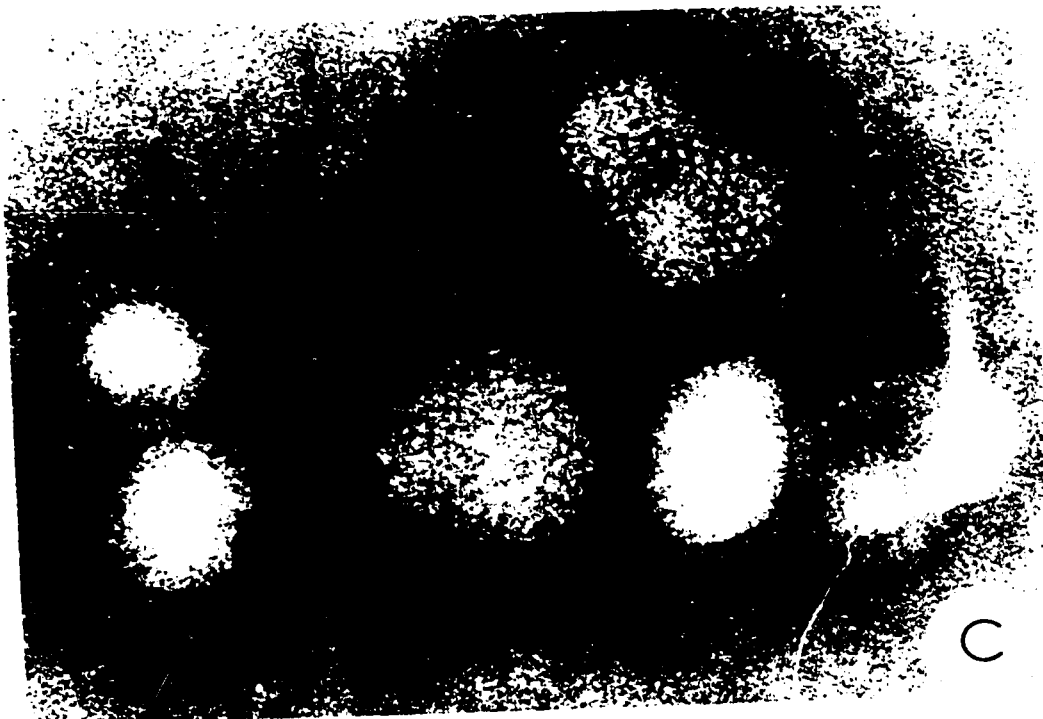
- A = Enterovirus
appr. x 249,000
- B = Adenovirus
appr. x 230,000
- C = Influenza virus
appr. x 235,000
- D = Reovirus
appr. x 249,000
- E = Vaccinia virus
appr. x 115,000
- F = Herpes simplex virus (empty particle)
appr. x 208,000
- G = Herpes simplex virus (full particle)
appr. x 200,000



A



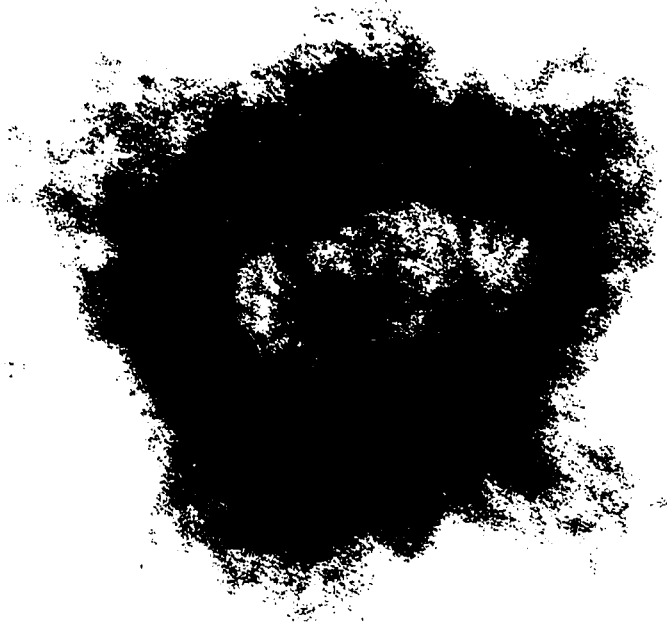
B



C



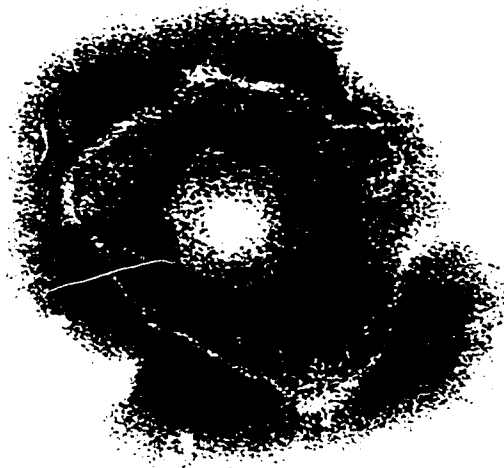
D



E



F



G

DISCUSSION

The results of the experiments indicate qualitatively and quantitatively that the sensitivity of pAGMK to herpes simplex and coxsackie B₄ viruses was affected when these cells were maintained at room temperature. Sensitivity of Vero cells did not seem too much affected and this is in agreement with the results of Doane and Anderson (1971).

On the basis of the above, one may be tempted to choose Vero cells as the cells of choice to be made available to hospitals for maintenance at room temperature ready for immediate inoculation. We felt however that the choice should be based also on relative sensitivity to various viruses. In this respect pAGMK proved to be more susceptible and more versatile than Vero cells. From the results, it is our feeling that both cell types should be used for maintenance at room temperature for immediate inoculation so that each would complement the other. It is of interest that coxsackie A9 grew on pAGMK but not on Vero cells as seen with both the clinical specimen and the laboratory strain. McLaren et.al. (1960) reported that continuous human cells lines were resistant to coxsackie A9 and commented that perhaps this could be characteristic of all primate cell lines.

The results of immediate inoculation of specimens into aged cells and inoculation of the same specimens after refrigeration is worth noting. Reports on virus isolations can be made earlier following direct inoculation as compared with inoculation after storage at refrigerator temperature. The results also indicate that, in field studies, specimens can be inoculated and held at room temperature without ill effects. This perhaps provides the diagnostic virology laboratory with the much needed transport

medium as suggested by Doane and Anderson (1971) which could keep viruses in their viable state. In this way the viruses can adsorb to the cells, penetrate and in some cases replicate while cells are being held prior to transportation to the laboratory for subsequent examination. (Farnham and Newton, 1959; Scott et.al., 1961; Van Kirk et.al., 1971)

The human lung cells were lost through freezer breakdown and, as a result, we were unable to determine the exact time hemadsorption would first appear in these cells following inoculation with influenza viruses. However, it is worth noting that hemadsorption was first observed on pAGMK with influenza PR8 virus 13 hours after inoculation (Sattar and Westwood, 1968). The 6 hrs. needed for cells to show hemadsorption in our experiments with lung cells is within the normal hours of a working day. Positive results could therefore be reported on the same day that cultures are received in the laboratory.

The use of the electron microscope allowed direct visualization of viral particles and this would be of diagnostic help in positive cases where tissue culture cytopathology is uncertain or misleading.

PART THREE

SIGNIFICANCE OF DIRECT HEMAGGLUTINATION(HA) WITH THROAT WASHING

INTRODUCTION

Direct examination of specimens had been used in the past in rapid diagnosis of myxovirus infections. Kalter (1950) reported on direct HA test with throat washings after these had been concentrated by adsorption onto and elution from erythrocytes. In 1965, Peizer identified influenza viruses from throat washings by ultracentrifuge concentration and micro HA assay on these specimens. Doane et.al. (1967) identified paramyxoviruses from nasopharyngeal secretions by direct examination with the electron microscope and also hemagglutination inhibition (HI) test. The use of direct hemagglutination with throat washing however is not mentioned by the standard texts of virology (Horsfall and Tamm, 1965; and Rhodes and van Rooyen, 1968). During an outbreak of influenza virus infection in 1970-71 we examined throat washings from patients and healthy individuals by direct hemagglutination to determine its significance in such specimens. Serologic studies were done on these patients to correlate the results. The throat washings were also examined by electron microscopy and the results of these studies are presented here.

MATERIALS AND METHODS

SPECIMENS

73 throat washings were obtained during the epidemic (Dec. 1970 - Feb. 1971) from patients attending the Family Practice Unit, Ottawa Civic Hospital, and these were inoculated immediately into tissue cultures for virus isolation studies. The specimens were then placed at 4°C overnight at the doctor's office before being sent to the laboratory. During the initial stages, the specimens after inoculation into tissue cultures were stored at -20°C until they could be tested for hemagglutination (HA). Later on, specimens were tested on the same day they were received in the laboratory.

Control throat washings were obtained during the epidemic from 13 persons who gave no history of illness. 26 other throat washings were obtained from patients during March, 1971. The throat washings from patients and controls were treated in the same manner.

Direct Hemagglutination Test

Throat washings were centrifuged at about 1800 rpm for 30 minutes to sediment mucous and any extraneous materials. The supernatant fluids (SNF) were taken and these were serially diluted with microdiluting loops in microplates. 0.025 ml volumes were used with phosphate buffered saline (PBS) as diluent. 0.5% guinea pig red blood cells (rbc) was added to each dilution in the cups and the mixture left at room temperature for 1 hour. Presence or absence of HA was recorded on the basis of erythrocyte pattern in the control.

Concentration of the throat washings by adsorption onto and elution from rbc as described by Kalter (1950) was attempted. 5 ml of throat washings was added to 2 ml of 1% guinea pig rbc and placed at 4°C for 1 hour. The

material was centrifuged and the SNF discarded. 0.5 ml saline was added to the sedimented cells. The cells were well suspended and incubated at 37°C for 2 hours with occasional shaking to permit elution to take place. The material was again centrifuged and supernatant fluid removed and tested for HA. This method was found to be unsuitable because of the occurrence of haemolysis and was therefore discontinued.

Serology

Paired sera were obtained from the patients and these were tested for complement fixing (CF) antibodies against influenza A and B; parainfluenza 1, 2, 3, SV₅, adenovirus, herpes simplex, Mycoplasma hominis and pneumoniae, respiratory syncytial virus and mumps. Hemagglutination inhibition and neutralization tests were also carried out.

In the CF test 3 different levels of antigen units for influenza and parainfluenza viruses were used. Ross et.al. (1964) had indicated that the use of different levels of antigen units could help determine early antibodies.

Removal of non-specific inhibitors from sera for HI tests was done as recommended by the WHO Committee on Respiratory Diseases (1959) and Schmidt and Lennette (1965). Potassium periodate was used for removal of influenza A and B inhibitors and cholera filtrate treatment for the parainfluenza viruses. (See Appendix)

Neutralization tests were carried out as described under Materials and Methods using influenza A2/Hong Kong, influenza B/Canada/66, parainfluenza 1 (Sendai) and parainfluenza 3. 100 TCID₅₀/0.1 ml of viruses were used. (See Part One Material and Methods)

Electron Microscopy (EM)

24 throat washings which were positive by hemagglutination test were examined. Three methods were employed in preparing the grids for examination by EM. The first method was the modification of the agar method of Kelen et.al. (1971) as described by Anderson and Doane (1971). Formvar-carbon coated grids were placed on 1% agar contained in microtitre cups. Drops of SNF from throat washings were placed on the grid and allowed to diffuse into the agar for 10 - 20 minutes. The grids were then removed from the agar and negatively stained using 2% solution of sodium phosphotungstate (PTA) pH 7.0.

A second specimen grid was prepared from a pellet obtained from throat washing (5 - 8 ml) by ultracentrifugation at 42,000 rpm for 2 hours, using Beckman Model L-2 ultracentrifuge. The pellet was suspended in 0.1 ml deionized water and one drop of this was added to a larger drop of water on parafilm (Doane et.al., 1969). A Formvar-carbon coated grid (400 mesh) was touched to the surface of the drop and stained with PTA. The grids were placed in small plastic petri dishes and allowed to dry in air. These were examined in an electron microscope at a magnification of about 45,000 times.

The third method involves preparation of grids using the re-suspended pellets obtained by ultracentrifugation of throat washings and the agar method of Anderson and Doane (1971). The coated grids were placed on the agar as previously described. A drop of the re-suspended pellet was placed on the grid and allowed to diffuse into the agar for 10 - 20 minutes. The grid was then stained with PTA, and examined by Philips EM300 electron microscope. (See p. 66 for details of electron microscopy)

RESULTS

Direct Hemagglutination With Throat Washings

73 throat washings were tested for HA agents during the three month period of the outbreak (Dec. 1970 - Feb. 1971). 24 of these were positive and regarded as presumptive influenza infections. The breakdown of the results were as follows: 10 positives during December, 1970, 8 in January and 6 in February, 1971 (Table 20). The hemagglutination was usually very weak and the titres never exceeded 1:2. As a result of this, no attempts were made to identify the agent by HI test.

Of the 13 specimens tested from healthy individuals only 1 was positive. None of the 26 throat washings tested during March, 1971 were positive for hemagglutinating agents.

Serology and Virus Isolation

Out of the 24 cases in which throat washings were positive for hemagglutinating agents, only 7 were confirmed as influenzal infections by complement fixation (CF) tests. 17 cases which were positive by hemagglutination were serologically negative and this gave a "false" positive rate of 71%. 13 cases with positive serologic diagnosis were negative by hemagglutination tests with throat washings. (Table 21)

9 hemagglutinating viruses were isolated from the 73 throat washings during the outbreak. 8 of these were confirmed by serology leaving only 1 case in which virus isolation was not confirmed by serology (Table 22). 2 throat washings which were positive for hemagglutinating agents were also positive by virus isolation and serology.

No serology tests were done on the 13 healthy individuals who gave specimens during the outbreak, since no paired sera were obtained.

There were no virus isolations made in this group.

26 patients also gave specimens during March, 1971 and none of these were positive by virus isolation or serology which indicated that the outbreak was confined to the three month period of December, 1970 to February, 1971.

Electron Microscopy (EM)

Direct examination of specimens by electron microscopy had been used by Doane et.al. (1967) and Joncas et.al. (1969) in diagnosis of virus infections of the respiratory tract. The authors examined nasopharyngeal secretions. Examination of throat washings by electron microscopy for virus particles had not been successful due to the low concentration of viruses in these specimens (Anderson and Doane, 1970). Hemagglutination occurs when virus concentration is about 10^6 particles/ml (Hirst, 1965) and one needs between 10^8 to 10^9 particles/ml in a preparation in order to detect viruses by electron microscopy. The 24 throat washings which were positive for hemagglutinating agents were therefore concentrated and examined by electron microscopy. Three concentration methods were used to prepare grids for examination, namely the agar method described by Anderson and Doane (1971), ultracentrifugation and a combination of ultracentrifugation and agar method. These methods have been described in detail in Materials and Methods. (p.89)

In spite of these concentration procedures, no virus particles were seen in any of the specimens. Influenza viruses had been isolated from 2 of the 24 specimens and this proved that viruses were present in those specimens yet no virus particles were seen when these specimens were examined by

electron microscopy. This suggests that although viruses could be present in the other specimens, the concentration procedures used were not able to bring the levels of viruses in the specimens within electron microscopy detectability. The maximum efficiency that could be attained by the two methods was 80 times for ultracentrifugation and about 100 times concentration with the agar method.

TABLE 20

BREAKDOWN OF POSITIVE MICRO-HEMAGGLUTINATION
WITH THROAT WASHINGS

	Number Positive	Number "False" Positive	% "False" Positive
December, 1970	10	5	50%
January, 1971	8	7	87.5%
February, 1971	6	5	83.3%
TOTAL	24	17	

TABLE 21

RESULTS OF DIRECT MICRO-HEMAGGLUTINATION
WITH THROAT WASHINGS
(DECEMBER 1970 - FEBRUARY 1971)

	DIRECT MICRO-HA WITH THROAT WASHINGS		
	POSITIVE	NEGATIVE	TOTAL
Serology			
(CF)			
Positive	7	13	20
Negative	17	36	53
TOTAL	24	49	73

% "False" positive = $17/24 \times 100 = 70.83\%$

TABLE 22

RESULTS OF TISSUE CULTURE ISOLATIONS
FROM THROAT WASHINGS
(DECEMBER 1970 - FEBRUARY 1971)

	TISSUE CULTURE ISOLATIONS		TOTAL
	POSITIVE	NEGATIVE	
Serology (CF)			
Positive	8	12	20
Negative	1	52	53
TOTAL	9	64	73

% "False" positive = $1/9 \times 100 = 11.1\%$

DISCUSSION

The throat washings were examined by direct hemagglutination method to determine the usefulness of such a method as a rapid diagnostic tool during an outbreak of upper respiratory tract infection. The high percentage of false positives threw considerable doubt on the value of this technique for rapid diagnosis of myxovirus infections. Kalter (1950) reported that in his studies 33.3% non specific HA positives were obtained with the throat washings. He stated that this might be due to activity of saliva or other agglutinating agents. Other workers have also reported instances where virus antigens could be demonstrated in specimens by virus isolation or immunofluorescence with nasal smears but infection could not be confirmed by serology or isolation (Liu, 1956, 1961; Kasel et.al., 1965; Andrews et.al., 1966; Ebisawa, et.al., 1968). Hence it is possible that the virus antigen or related substance was present in the specimen but infection was not confirmed by conventional methods of isolation and serology.

Possible sources of "false" positives could be due to the fact that serologic diagnosis was not possible because the patients were seen very late during the course of infection. Our analysis of the 17 "false" positive cases however showed that 13 of these patients were seen before the 5th day after onset of infection which is well within the period when serologic diagnosis could be possible. Also as many as 12 of these patients had antibody levels of less than 1:8 to influenza and parainfluenza viruses.

The throat washings were tested after centrifugation at 1800 rpm to remove mucoid and extraneous materials which could produce non-specific hemagglutination. We also used guinea pig rbc not older than 7 days after

receipt for the hemagglutination tests, thus eliminating spontaneous agglutination due to older cells. Another possible source of "false" positive results could be that an agent(s) which would cause respiratory infection and also hemagglutinate rbc was excluded in our battery of antigens.

We used three different optimal units of influenza and parainfluenza virus antigens in the CF tests as suggested by Ross et.al. (1964). This was done in order to increase the sensitivity of the tests and to enable us to detect early antibodies which might be missed with lower levels of antigens. Although the sensitivity of the tests was increased as shown by higher titres of antibodies obtained with the higher units of antigens, no diagnostic four-fold rises were detected with the paired sera. The electron microscopy studies also failed to reveal any virus particles in the throat washings but this might have been due to the agent being present in the throat washings in low concentration.

One suggestive fact which emerged from our studies is the high proportion of positive hemagglutination with throat washings from patients during the epidemic period as compared to the controls. The detection of hemagglutinating agents in throat washings coincided with the peaks of virus isolations and serology and were absent in March when no viruses were isolated and no serologic diagnostic rises were obtained. This may suggest a cause and effect relationship between the presence of hemagglutinating agents in throat washings and an incidence of influenza-like infection. The potential use of such a simple method as a possible signal of an outbreak needs to be examined further. This technique at this stage could not however be relied on for rapid diagnosis of myxovirus infections.

PART FOUR

ADAPTATION OF THE POLYELECTROLYTES (PE60) METHOD OF CONCENTRATING VIRUSES FOR ROUTINE LABORATORY USE

INTRODUCTION

With the advent of virus chemotherapy there would be a great demand for rapid diagnosis of virus infections. Rapidity of reporting on virus isolates is determined by how quickly virus activity can be detected in cell culture and this is determined in large measure by the quantity of virus present in the inoculum. The low concentration of viruses in stool (Melnick, 1965) requires that these specimens should be concentrated. A method of concentrating viruses from sewage and human excreta by adsorption on and elution from polyelectrolytes (PE60) had been described by Wallis et.al. (1969, 1971). Chaudhary and Westwood (1972) evaluated PE60 in concentrating enteroviruses from stool for subsequent electron microscope studies. The methods employed by all these workers involved suspending the stool in saline solution and adjusting the pH to optimal levels using a pH meter. However the constant use of a pH meter could be both cumbersome and unpleasant in the routine laboratory where many specimens may require to be processed.

This study was therefore performed to examine the possible use of buffer solution as suspending medium for stool. Also the pH was checked by indicator instead of pH meter and the use of other eluting solutions apart from the recommended buffer was examined.

MATERIALS AND METHODS

Viruses:

Echovirus type 9 and coxsackievirus B₄ were used for inoculation of stool suspensions. These viruses had been isolated from stool specimens at the Virus Diagnostic Unit, Ottawa Civic Hospital, Ottawa, Canada and passaged in primary African green monkey kidney cells.

Tissue Culture:

Primary African green monkey kidney (pAGMK) cells were used. The source and method of handling these cultures had been described in part one.

Virus Assay:

Virus titres were determined by titration in pAGMK culture tubes. 1 log. dilutions were made and inoculated into 4 tubes per dilution. Cell cultures were incubated at 37°C for 7 days and cytopathic effect (CPE) recorded. Virus titre was calculated by Kärber's method.

Preparation of PE60:

An insoluble cross-linked copolymer of isobutylene maleic anhydride identified as PE60 was used. This was kindly supplied in the form of 100 mesh powder by The Monsanto Co., St. Louis, Mo. It was freshly prepared for use as recommended by Wallis et.al. (1969). PE60 was suspended in distilled water and shaken vigorously for 30 minutes. The suspension was centrifuged at 1800 rpm for 5 minutes. The supernatant fluid was discarded and the packed PE60 was washed twice with distilled water. Enough distilled water was added to resuspend the PE60. The suspension was distributed into tubes such that 30 mg of PE60 was contained in each tube. The tubes were centrifuged and supernatant fluids discarded. The packed PE60 was used for virus adsorption.

Determination of Optimal pH for Virus Adsorption

Stool specimens were obtained from healthy persons. 10% suspensions were prepared in saline and clarified by centrifugation at 1800 rpm for 30 minutes. 9 ml portions of clarified suspensions were added to the prepared PE60 (30 mg) in tubes. 1 ml of a known titre of virus was added to the stool suspension/PE60 mixtures. Predetermined number of drops of 0.1N HCl or 0.1N NaOH were added to obtain different pH levels (3.5 to 7.0). The final pH was checked by adding a few drops of the mixtures to equal volumes of universal indicator in tubes and matched with standard tubes. The stool/PE60/virus mixtures were held at room temperature with occasional shaking for 1 hr. 30 minutes to effect virus adsorption. The tubes were centrifuged at 1800 rpm for 5 minutes. The supernatant fluids were assayed for unabsorbed virus.

Control suspension seeded with virus but not treated with PE60 was assayed in parallel.

Standard pH Tubes:

Citrate/phosphate and phosphate buffers of varying pH range (3.5 to 8.5) were prepared (see appendix). Equal number of drops of the buffers were added to equal volumes of universal indicator in tubes and these served as standards. Fresh tubes were prepared for each set of experiments.

Optimal pH for Virus Elution:

The sedimented PE60 from virus adsorption at pH 5.0 was suspended in 2 ml of eluent (10% fetal calf serum in Sorensen's buffer) at pH 8.0, 8.5 and 9.0. 0.1N NaOH was used to adjust pH of the suspension to the original levels. The tubes were centrifuged at 1800 rpm for 5 minutes and the supernatant fluids titrated for eluted virus.

Buffer as Suspending Medium for Stool:

0.2M citrate/phosphate buffer at pH 5.0 was used as suspending solution for stool. 9 ml of such suspension was added to prepared PE60 and 1 ml of virus. The pH was checked as a precautionary step with the indicator. Virus adsorption and elution were done as previously described. Supernatant fluids after adsorption and elution were titrated for virus.

RESULTS

pH of Adsorption:

The optimal pH of adsorption of virus by polyelectrolytes was determined using 7 different pH levels ranging from 3.5 to 7.0. Adsorption at each pH was tested at least 3 times. Different pH levels had little effect on the adsorption of echovirus type 9 (Table 2B). Optimal adsorption was obtained with pH 5.0 and 5.5 (Fig. 6). pH 5.0 was selected for subsequent adsorption experiments.

Elution of Virus:

Efficient elution of adsorbed virus from the polyelectrolytes depends on the pH of eluent and this is usually in the alkaline range. (Wallis et.al., 1969) Experiments were therefore performed to determine the optimal pH for elution using the buffer suggested by these workers. Poor elution was obtained when the 0.05M Sorensen's buffer was used. (Table 24) The best elution (30%) with this molarity was obtained at pH 8.5 with 2 ml of eluent. When the same buffer was used at higher molarity elution was enhanced many times. 0.1M Sorensen's buffer eluted an average of 66% of the adsorbed virus at pH 8.0 and 70% at pH 8.5. The same elution efficiency was obtained with 0.1M Na_2HPO_4 at pH 8.6. There is no significant difference between these elution values when analysed statistically by t-test at the 5% level.

Buffer as Suspending Solution for Stool:

Preliminary experiments showed that when stool was suspended in 0.1M citrate buffer the original pH 5.0 was altered. However at 0.2M, the buffering effect was maintained when stool suspension was made and PE60

added. We therefore used that molarity for our experiments. Table 25 shows that echovirus type 9 and coxsackievirus B₄ could be adsorbed efficiently in the presence of the high concentration of salts. Elution was also efficient with 0.1M Na₂HPO₄.

TABLE 23

DETERMINATION OF OPTIMAL pH FOR ADSORPTION OF VIRUS (ECHOVIRUS TYPE 9)
FROM STOOL SUSPENSION USING PE60

	EXPT. 1		2		3		AVERAGE % OF ADSORPTION
	LOG TITRE/0.1 ml ADSORBED	% ADSORBED	TITRE	% ADSORBED	TITRE	% ADSORBED	
Control - Virus in PBS	4.8		4.5		4.3		
PE 60/Virus/stool SNF							97.2
pH	3.5	97	2.0	99.7	3.0	95.0	
	4.0	84	1.8	99.8	2.8	97	93.6
	4.5	99	<1.8	>99.8	2.3	99.9	99.6
	5.0	99.9	<1.8	>99.8	<2.0	>99.9	99.9
	5.5	>99.9	<1.8	>99.8	<2.0	>99.9	99.9
	6.0	99.8	1.8	99.8	2.3	99.9	99.8
	7.0	99.9	1.8	99.8	2.0	99.5	99.7

Fig. 6

DETERMINATION OF OPTIMAL pH OF ADSORPTION OF
ECHOVIRUS TYPE 9 FROM STOOL SUSPENSION BY PE 60

pH OF FAECAL / PE 60 SUSPENSION IN SALINE .

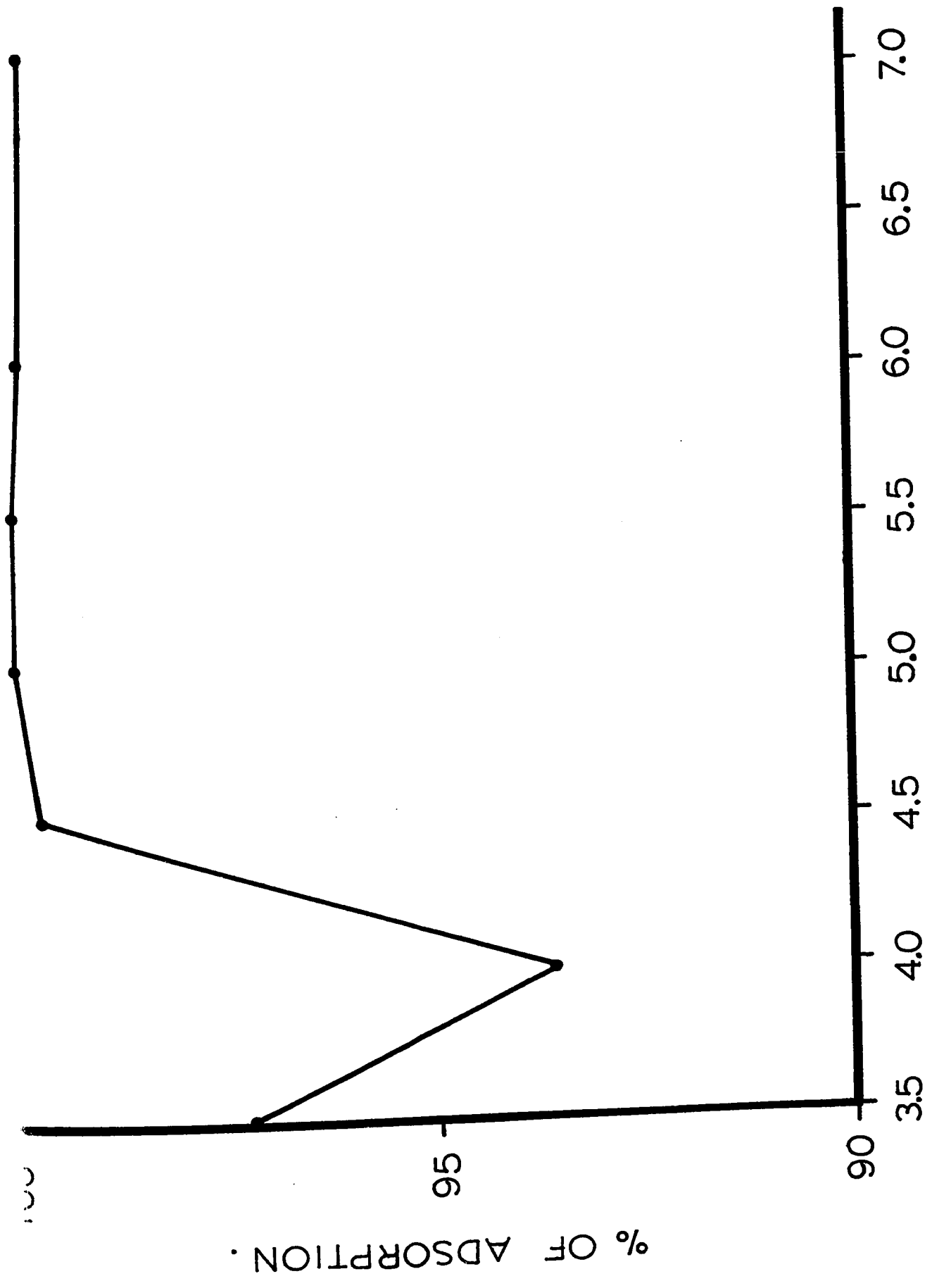


TABLE 24

ELUTION OF ADSORBED ECHOVIRUS TYPE 9 FROM PE60

Buffer	Molarity	pH of Elution	Volume of Eluent	Number of Experiments	Average Elution % of Adsorbed virus
Sorensen's	0.05	8.0	1 ml.	5	7
"	0.05	8.0	2 ml.	3	9
"	0.05	8.5	1 ml.	5	8
"	0.05	8.5	2 ml.	1	30
"	0.05	9.0	1 ml.	3	0
Sorensen's	0.1	8.0	2 ml.	2	66
"	0.1	8.5	2 ml.	3	70.5
Na ₂ HPO ₄	0.1	8.6	2 ml.	6	70.0

TABLE 25

RESULTS OF EXPERIMENTS USING CITRATE/PHOSPHATE BUFFER AS
SUSPENDING MEDIUM FOR STOOL AND SUBSEQUENT CONCENTRATION OF VIRUS
FROM SUSPENSION WITH PE60

Molarity of Suspending Buffer	pH of Adsorp- tion	Virus Type	% of Adsorption			% of Elution with 0.1M Na ₂ HPO ₄ (2 ml)		
			1	2	3	1	2	3
0.2 M	5.0	Echovirus Type 9	69	69	69	70	70	77
0.2 M	5.0	Coxsackie virus type B4	69	69		93	91	

DISCUSSION

This report confirms the results of Wallis et.al. (1969) that PE60 could be used for concentration of viruses from stool specimens. It also demonstrates that a simple method of testing the pH by spot test with indicator could produce equally efficient results of virus adsorption. Our results of virus adsorption showed a very broad pH range which is in contrast to the results of Wallis et.al. (1971) and Chaudhary and Westwood (1972) who had relatively little adsorption at pH 4.0 and 7.0. This can be explained by the precise nature of pH attained by these workers who used pH meter to measure pH values. We used an indicator system. The higher percentages of adsorption (99.9%) at our optimal pH was similar to the value (99.9%) obtained by Wallis et.al. (1971).

There was however a drop in percentage of adsorbed virus as the PE60 powder aged through storage and this was reflected in the results of subsequent experiments using both saline and buffer solutions. Wallis et.al. (1969) reported that storage of the powder did not seem to affect adsorption; but this is likely to vary from lot to lot. In fact, Chaudhary and Westwood (1972) obtained 85% adsorption in their earlier experiments and only 69% in later attempts with clinical specimens. They were not able to explain this drop but the results of our experiments suggest that the adsorption of viruses by the lot of PE60 we used, was similarly affected. It is therefore necessary as suggested by Wallis et.al. (1969) to test the powder at intervals to ascertain its stability.

The viruses used were adsorbed efficiently in buffer solution and the use of a strong buffer system as described in this report takes away the

unpleasant chore of adjusting pH of stool suspensions. Our results of elution of virus indicated that although the eluent recommended by Wallis et.al. (1971) could be used, a higher molarity was required for efficient elution. This finding is similar to the results of Chaudhary and Westwood (1972) who also obtained the best elution with 0.1 molar solution instead of 0.05M solution. We found also that both the single phosphate and double phosphate buffer could be used for efficient elution of virus.

In our experiments although equal adsorption was attained the average percentage of elution of echovirus type 9 was 72.3 and for coxsackie B₄ was 92% (Table 25). There was significant difference when these results were tested by ~~t-tests~~ at the 5% level. Wallis et.al. (1971) also reported 10 times concentration for coxsackieviruses and between 6 and 8 times concentration for echoviruses. It appears from these results and ours that coxsackieviruses could be eluted more efficiently from PE60. The selective adsorption of viruses on PE60 could be advantageous where toxicity of specimens is involved. We noticed that in seven cases when control tubes inoculated with stool suspension showed degeneration as a result of "toxicity" and contamination, tubes inoculated with eluted virus were not contaminated nor degenerated. The method described here could be extended and applied in the laboratory in special cases for autopsy specimens. These specimens apart from being least productive because of low virus concentration (Herrmann, 1972; Schmidt, 1972) are also usually toxic for tissue culture. The success in increased virus isolation from this type of material which probably carries more weight in diagnosis could help elucidate possible virus implication in some diseases.

PART FIVE

COMPARISON BETWEEN VENOUS AND CAPILLARY BLOOD FOR COMPLEMENT FIXING ANTIBODY TITRES AND IMMUNOGLOBULINS

INTRODUCTION

Serologic information is useful in obtaining virus diagnosis when virus isolation is not possible. Such data is also very useful in epidemiology. Venous puncture has been the conventional method of obtaining blood samples for serology. Two samples of blood are required for serologic diagnosis namely the acute and convalescent samples. The taking of venous blood particularly the convalescent sample when the patient has recovered causes difficulties, and when infants are involved this method has been found to be intolerable. Many workers have therefore used finger puncture method. (Beem et.al., 1960; Plotkin et.al., 1968; Herrmann et.al., 1971; and Maletzky et.al., 1971). During our studies on virus diagnosis in community practice our analysis showed that serological diagnosis yields the great majority of the positive diagnostic results and to obtain maximum diagnosis of virus infection, the specimens taken should include paired sera. Since infants were included in our study we decided to use the finger puncture method of obtaining blood samples from these patients because it is less traumatic.

A survey of the literature pertaining to venous and capillary blood presented us with different methods of assessing the results and sometimes conflicting reports. For example, Beem et.al. (1960) obtained 0.1 ml of capillary blood and diluted this to 1 ml. The clot and cells were removed and the supernatant fluid was considered as 1:20 dilution. Plotkin et.al. (1968)

collected 0.1 ml of blood from the fingertip directly into 0.9 ml saline. The supernatant fluid was referred to as 1:10 dilution. Allansmith et.al. (1968) reported that using the single radial immunodiffusion method, they found that immunoglobulin value in finger puncture sera and venipuncture sera were the same. They however did not indicate how sera were diluted.

With these reports in the background we embarked on a study to compare the complement fixing antibody levels in sera obtained from venous and capillary blood. Adult patients attending the clinic were persuaded to give both venous and capillary blood and these were titrated in parallel. Some of these blood samples were also examined for their immunoglobulin titres using the double immunodiffusion agar method.

MATERIALS AND METHODS

Blood Specimens:

a) Venous blood: This was collected by obtaining 10 ml blood into tubes from the basilic vein of the hand using sterile disposable syringe. The blood was allowed to clot and the serum obtained by centrifugation. The specimens were stored at -20°C until tested. 1:4 dilutions were prepared using Veronal buffer. Sera were inactivated at 56°C for 30 minutes before use.

b) Capillary blood: The thumb was punctured and 0.2 ml blood collected using blood diluting pipette, into 0.6 ml Veronal buffer. The clot and cells were sedimented by centrifugation and the supernatant fluid collected and stored at -20°C . The serum was considered as 1:8 dilution. Sera were thawed and inactivated at 56°C for 30 minutes before use.

Complement Fixation (CF) Test:

Standard microtechnique as described in part one (materials and methods) was used. 54 sera were titrated using the following antigens: influenza A, B, (soluble), herpes simplex, respiratory syncytial virus, adenovirus, mumps S and V, parainfluenza 1, 2, 3, Mycoplasma hominis and pneumoniae.

Immunodiffusion:

The double microdiffusion method in agar was used. Immunodiffusion discs were purchased from the Research Products Division, Miles Laboratory Inc., Illinois. The diameter of the plates was 6 cm and consisted of a central well and six outer wells. The constituents of the agar plate were as follows:
Agarose 0.9%; pH 8.5.

Buffer system	Borate-saline
Ionicity	0.175
Preservative	0.01% Methiolate
Indicator Dye	Trypan Blue

Goat antisera to human IgG and IgM were purchased from Hyland, Division of Travenol Laboratories Inc., Costa Mesa, Calif., USA.

The central wells were filled with antisera to human IgG and IgM and dilutions of the venous and capillary sera were put in the outer wells. Samples from the same individual were tested on the same day and on the same plate. The plates were incubated at room temperature in a moist container and examined for precipitin each day for 3 days. The titre was taken as the reciprocal of the highest dilution of serum producing precipitation within 3 days.

RESULTS

Complement Fixing Antibody Levels:

54 blood samples were obtained from 27 patients, each patient giving both venous and finger puncture blood at the same time. These samples were titrated in parallel for complement fixing antibody levels by the microtechnique. Twelve antigens were used and yielded a total of 308 individual results which could be compared, excluding those sera where negative results were obtained with all the antigens in both venous and corresponding finger puncture blood. The finger puncture samples were obtained by taking 0.2 ml of whole blood into 0.6 ml diluent. The mixtures were centrifuged to remove clots and cells, and the supernatant fluids were used in the titrations. Because of this procedure of obtaining the samples, the dilution factor was doubled, assuming a haematocrit of 50%. The antibody titres of the venous blood samples were therefore compared with twice the antibody titres in corresponding finger puncture blood and these comparisons are seen in Table 26. In 80 instances in which titres higher than 1:16 were obtained, only in 11 cases did finger puncture samples show titres higher than venous samples. In 60 cases venous blood titres were higher by two-fold or more. In spite of this, when the results were tested statistically by t-test, the differences in titres were not significant at the 5% level.

The antibody titres obtained in finger puncture samples were qualitatively similar to those found in venous blood from the same individual. In four cases however when diagnostic rises against herpes simplex antigen were obtained with venous blood, two finger puncture blood samples failed to show the four-fold rise.

The higher titres obtained with venous blood samples is further illustrated in Fig. 7 where more of the points are found closer to the venous blood axis.

Immunoglobulin Concentration:

Owing to the very small samples of capillary blood available it was decided to use an immunological assay for this experiment. Table 27 shows the results as determined by double immunodiffusion in agar. IgG and IgM titres in venous blood were consistently higher than in capillary blood. This may explain the higher CF titres obtained in venous blood. Six sets of sera were tested and in 3 cases, IgG levels in venous blood were four-fold higher than in capillary blood. IgM levels in both types of blood were always lower than IgG levels and this was expected.

TABLE 26

COMPARISON BETWEEN CF ANTIBODY TITRES
IN BLOOD OBTAINED SIMULTANEOUSLY FROM PATIENTS
BY VENOUS AND FINGER TIP PUNCTURE(CAPILLARY)

		VENOUS BLOOD ANTIBODY TITRE*				TOTAL
		<16	16	32	>64	
CAPILLARY BLOOD ANTIBODY TITRE*	<16	228 ^a	34	7	0	269
	16	11	4	15	2	32
	32	0	0	1	2	3
	>64	0	0	0	4	4
TOTAL		239	38	23	8	308

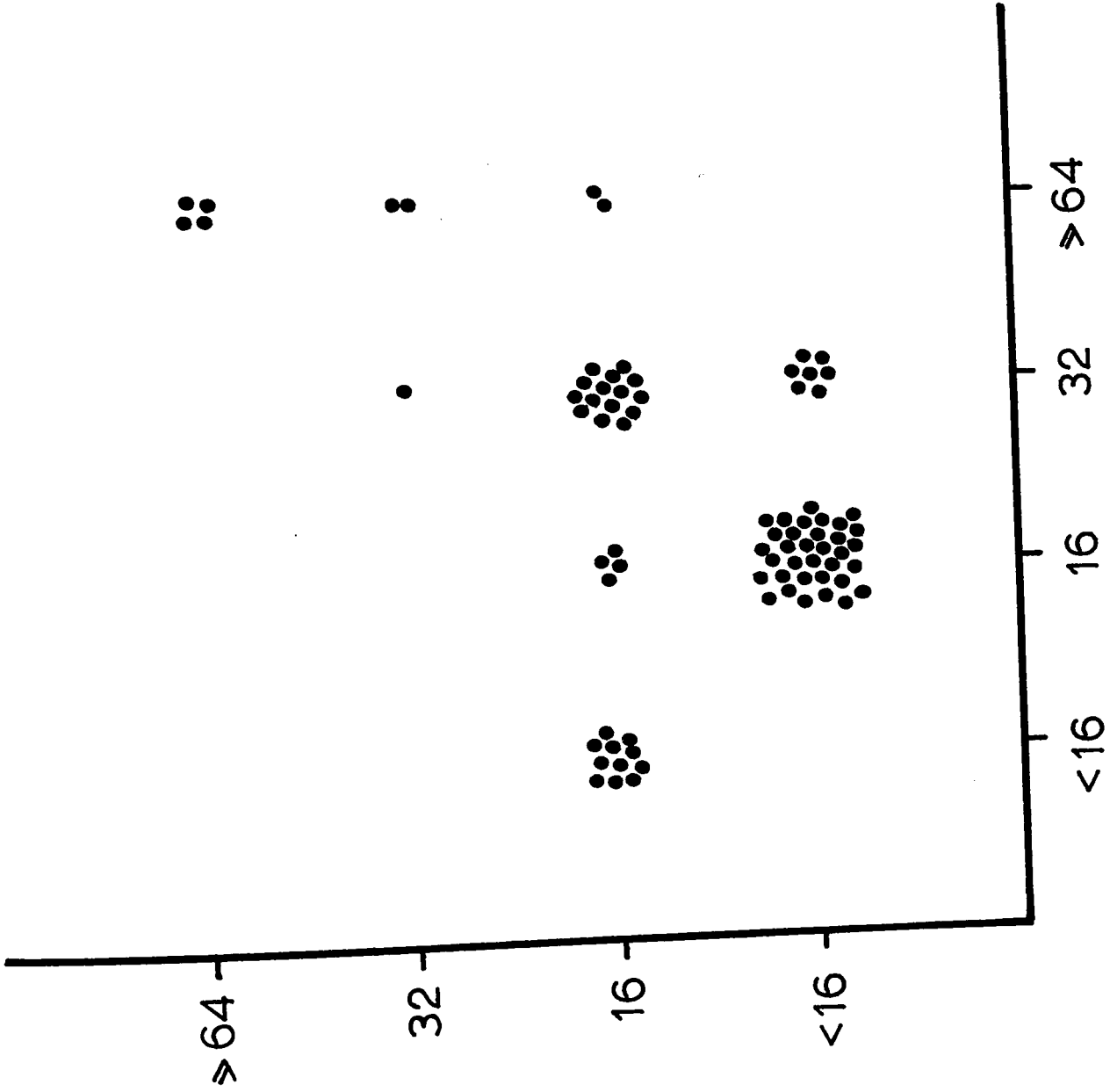
* = Reciprocal of serum dilution positive

a = Number of times corresponding titre was obtained

Fig. 7.

COMPARISON BETWEEN COMPLEMENT FIXING ANTIBODY TITRES IN BLOOD OBTAINED
SIMULTANEOUSLY FROM PATIENTS BY VENOUS AND FINGER TIP PUNCTURE (CAPILLARY)

CF ANTIBODY LEVEL IN CAPILLARY BLOOD



CF ANTIBODY LEVEL IN VENOUS BLOOD.

TABLE 27

IMMUNOGLOBULIN LEVELS IN VENOUS
AND CAPILLARY BLOOD (FINGER PUNCTURE)

PATIENT NO.	<u>Ig G Titre</u>		<u>Ig M Titre</u>	
	VENOUS	CAPILLARY	VENOUS	CAPILLARY
VP. 364	128*	32	>8	<8
VP. 368	32	8	<8	<8
VP. 369	>8	8	>8	<8
VP. 370	128	32	8	<8
VP. 376	>16	16	8	<8
VP. 388	>16	8	<8	<8

* = Reciprocal of serum dilution producing observable line of precipitation

DISCUSSION

Although the differences in complement fixing antibody titres were not statistically significant, we feel that such differences in titre should be borne in mind. The dilution factor with capillary blood was doubled as done by Beem et.al. (1960) and Gupta et.al. (1971) assuming a haematocrit of 50%. This gave a better correlation between venous blood and its corresponding capillary sample than direct comparison would give and such correction may resolve some of the contradictory results found in the literature. For example Cloonan et.al. (1970) reported that 18% of babies they studied had detectable rubella HI antibody at 6 months and 5% at 9 months. They did not specify how blood samples were taken. But Herrmann et.al. (1971) did not detect HI antibody 6 months after birth using finger puncture blood.

Plotkin et.al. (1968) found that in 9/24 (37.5%) cases rubella HI antibody in venous blood were higher than in capillary blood. In our results, in 60/80 (75%) instances venous blood gave higher CF titres than capillary blood.

Our results showed that immunoglobulin levels in venous blood are higher than in finger puncture blood and this is in divergence to the results of Allansmith et.al. (1969) who reported that the levels were the same. The determination of immunoglobulin levels in our case was carried out by the double immunodiffusion method which is more sensitive than the single radial immunodiffusion used by these workers (Crowle, 1961). This may explain the difference in our results.

We have shown that venipuncture serum has higher titre than finger puncture serum even when these are taken simultaneously. Comparison between

such two samples taken on different occasions and compared directly is likely to show even greater differences in titres. Negative serological diagnosis of congenital rubella is indicated by the ability of the affected infant's antibody titre to decline in a normal manner following birth (Hardy et.al. 1969). This is done usually by comparing cord serum and finger puncture serum; the former taken at birth and the latter taken 6 months after birth. Such comparison is therefore likely to show a fall in titre which could produce false results in diagnosis of congenital rubella. For epidemiological work many infants with low levels of immunity may be missed. Also this can affect vaccination schedules since maximum take of vaccine may not be achieved due to undetectable passive antibody.

Data on immunoglobulin levels are also used in medicine to diagnose a disease state or to predict unusual susceptibility to infection and in infants the finger puncture method had been used (Huntley and Lyerly, 1963; West et.al., 1962; Allansmith et.al., 1968). This procedure is also subject to errors.

In our opinion the findings reported here need further careful study since their implications are considerable in the context of paediatric diagnostic technique.

GENERAL DISCUSSION

This project was started to determine the effectiveness of a Virus Diagnostic Service working closely with clinicians and its usefulness in community medicine. It has been the prevailing opinion that virus diagnostic techniques are too expensive, time consuming and that the number of positives is too low. The period before results are reported is also too long to be of practical importance in patient care. In view of such remarks, it was found necessary to determine also the optimal methods of handling and examination of specimens so as to produce rapid diagnosis and the highest possible virus diagnostic rates.

Most virus infections are relatively mild, self-limiting and without sequelae. As a result of that they do not usually reach the hospital unless complications occur. By the time this occurs, the illness is often so far advanced as to be beyond the optimal period for virus isolation. This delay before patients are seen is further complicated by the many processes through which specimens are ordered, taken and finally transported to the laboratory. Such has been the dilemma of virus diagnosis and hence the low diagnostic rates obtained in the routine laboratory. Virus isolation rates would be improved therefore if the virus diagnostic laboratory could be taken to the community where the majority of virus infections are found and where the majority of acute infections are of viral aetiology. A set-up on the basis of the Family Practice Unit provided us with the means of bringing the laboratory to the community. And to determine the effectiveness of this system, the diagnostic rates of the Unit and the routine laboratory were compared.

To some extent our expectation was realized. In both virus isolation

and serologic diagnostic rates, the percentages of positives were higher in the Family Practice Unit investigations than in the routine laboratory (Tables 4 and 5). The overall diagnostic rate of 33.3% seems acceptable but the virus isolation rate of 10% is far below expectation. It must be mentioned here that virus isolation rates could be influenced to a great extent by the selection of patients. A higher percentage of success would be achieved if the study is restricted to a particular virus infection or to a particular epidemic. Pearson et.al. (1972) achieved an isolation rate of 34% by restricting their studies to only enterovirus infections and their percentage was even increased to 84% during the epidemic period. In general practice where virus studies are ordered as a routine, many infections of non-viral nature would be included which can reduce the percentage positives. The situation in the Family Practice Unit investigation was essentially the midway point between these two extremes because all cases of probable virus aetiology were studied and this was carried out throughout the year. The serologic results gave an indication of the extent of successful virus isolation as well as the degree of accuracy of the selection of patients. About 77% (231 out of 301) of the patients were seen before the 5th day which was well within the period when positive serologic diagnosis is possible yet only 33.3% overall diagnostic rate was achieved. This suggests that a large proportion of the patients clinically diagnosed as being infected with viruses were not, as determined by the methods available to us. This would influence our virus isolation rate which turned out to be low. Such negative results could however be useful to the physicians since it would help them to re-appraise their criteria for

clinical diagnosis of virus infections.

From our studies it had been shown that for optimal isolation of viruses, specimens should be inoculated into tissue culture on the day on which they are taken. This can be possible if a daily collection service of high efficiency is instituted. This is bound to meet with some difficulties since doctors' office hours tend to continue after full services of the diagnostic laboratory have closed down for the evening. The alternative would be to provide tissue cultures for direct inoculation within the doctors' office or at a satellite laboratory. The inoculated cultures can then be transported to the Central laboratory where they can be further examined. In a city like Ottawa such a system could be established, debarring political implications, by making use of the already existing private laboratories which are mostly located in medical buildings. There would however be an anxiety of possible abuse of such a service through indiscriminate ordering of tests since it would be easily accessible. In our experience, this would not be the case because during the study at the Family Practice Unit some of the clinicians had to be reminded from time to time to send specimens, although they were aware of the services.

The results with the primary African green monkey kidney cells and the Vero cells held at room temperature suggested that such treatment affected the sensitivity of the cells to virus and this could not be predicted. However, in field studies where immediate transportation is not possible, specimens could be inoculated into cell cultures which have been held at room temperature before and after inoculation, as suggested also by Doane and Anderson (1971).

Such a procedure was found to be more productive than holding of specimens at 4°C for a period of time before they are sent to the Laboratory for inoculation. Storage of cells at room temperature for immediate inoculation would find meaningful application in the developing countries where laboratory facilities are very limited especially in the tropical and sub-tropical countries. It can be assumed that since room temperature in these countries tends to be higher than in temperate countries, the effect of holding tissue cultures at room temperature may be minimal in such instances. Tissue cultures could be therefore held at room temperature and used successfully for virus isolation. This would eliminate the high cost of refrigeration of specimens prior to transportation to the Central laboratory. Cultures could be easily supplied to the regional centres which in most cases are linked by air routes to the capitals where the central laboratories are located. This would help in setting up a more efficient system for virus studies.

It is reasonable to speculate that through such a system many more of the unknown viruses in these tropical countries would be isolated and studied. It may be noted here that improvements in communication systems could allow one to be infected with an unknown agent for example in Africa, and then become sick with a contagious illness thousands of miles away which could be a medical problem. The example of the highly contagious and virulent Lassa virus from Nigeria is a situation to be remembered and this demands a more intensive system of studying these unknown viruses.

Although the speed of reporting of virus isolations was better in the Family Practice Unit investigations, only 50% of these were reported by

the fourth day. In this respect the value of laboratory diagnosis to the clinician in determining the treatment in individual cases is not very promising. On the other hand, the background information provided through the virus diagnostic services regarding virus infection prevalent in the community was of great value in influencing diagnosis. During the 1970-71 influenza episodes, virus isolation was made in early December and this alerted physicians to its presence in the community 3 weeks before its presence became obvious. The knowledge that an illness is of viral origin may lead to curtailment of the use of antibiotics which could spare the patient the cost of medicines and prevent the possible danger of drug reaction. The knowledge also of viruses prevalent in the community could be an aid in clinical diagnosis and management of patients. Pearson et.al. (1972) reported that from their studies on the impact of viral diagnosis on medical practice it became clear "that unnecessary hospitalization could be avoided when physicians were supplied with exact viral diagnostic information, even belatedly. This service is a financial asset, not a liability, when viewed in the overall context of the cost of providing health care." It is of special interest here that although the direct microhemmagglutination tests with throat washings did not seem to be diagnostically reliable in its present form, it might perhaps be useful as a possible signal of an outbreak of influenza infection. This method therefore needs further trials and also further studies to reduce the number of "false" positives as seen in our results.

The availability of the electron microscope as an aid in laboratory diagnosis would drastically change the present picture of virus diagnosis as

demonstrated by Doane et.al. (1967). These workers examined nasopharyngeal secretions directly under the electron microscope and remarked that the speed of reporting virus diagnosis could be reduced to within hours as against 2-7 days which is the average period required for identification of viruses by tissue culture techniques. They have subsequently shown that direct examination of other specimens like vesicular fluids, pustular materials and cerebrospinal fluid is also possible. Tissue specimens from biopsy or autopsy can be examined by preparing lysed tissue suspensions for examination by electron microscopy. Another area in which the application of the electron microscope has been useful is the examination of materials from inoculated cultures in which identification of virus is not definite by cytopathology. These procedures have all been found to reduce the period before virus identification is made by tissue culture techniques. Unfortunately, the electron microscope was not available to us during the greater part of this project as a result we were unable to assess how drastically its application could affect the speed of reporting our virus isolates. We however used the electron microscope in direct examination of throat washings which was not very successful.

The polyelectrolytes (PE60) concentration method first described by Wallis et.al. (1969) has been found useful in direct examination of stool specimens by electron microscopy for virus particles (Chaudhary and Westwood, 1972). The modification described in this report could make this method easier and more appealing for use. This method can be applied in public health projects such as monitoring of river water which in many tropical countries could be heavily contaminated with viruses due to lack of adequate sewage treatment.

We have shown that submission of more specimens from a patient increases the likelihood of obtaining positive virus diagnosis. The simplest procedure would be to submit throat washings or swab or any other relevant swab and paired sera. Throat washings or swabs are no problem but the taking of venous blood particularly the convalescent sample when the patient has recovered, causes difficulties. This is more so even with acute samples when infants are concerned and because of this, finger puncture method is preferred. The finger puncture sampling can be made satisfactory and relatively comparable to the venous sampling by doubling the dilution factor.

Introduction of country-wide vaccination campaigns in tropical Africa require that the natural immunity status of the population should be known. The use of a simple method of obtaining blood by finger puncture would not only be easily acceptable but would cut down on cost of obtaining syringes. The needles used for the finger puncture can be easily sterilized by flaming with alcohol and re-used.

It is our conclusion that a community virus service like that of the Family Practice Unit investigation could keep physicians informed as to the viruses which they are to encounter in relation to particular disease entities in the community. Valuable epidemiological information would also be gained through such a service. The availability of virus chemotherapy would no doubt keep many physicians more interested in laboratory diagnosis of virus infections, hence the success of such a service.

SUMMARY

1. A virus diagnostic service working in close collaboration with clinicians has been shown to be effective and useful in providing information about viruses prevalent in the community and influenced patient care. Virus isolations and serologic diagnostic results were obtainable more readily and earlier through this service than in the routine laboratory (Part One).

2. Storage of specimens at 4°C affected the outcome of virus isolation. The effect of storage of cell cultures at room temperature on their sensitivities was also demonstrated. It was clear from the experiments that the effects of storage of both tissue culture and specimens for virus isolation depended on the individual cells and the individual virus and could not be predicted. Therefore all specimens should preferably be transported to the laboratory on the same day and inoculated immediately in cells continually held at 37°C. In field studies however where immediate transportation is not possible, specimens could be inoculated into cell cultures which have been held at room temperature before and after inoculation. (Parts One and Two)

3. The results of direct hemagglutination tests with throat washings suggested that this technique could not be used satisfactorily at its present stage for virus diagnosis. It might however be useful as a signal of an outbreak of influenzal infections. (Part Three)

4. The usefulness of polyelectrolytes (PE60) for concentration of viruses had been confirmed. Adaptation of this method for routine laboratory use had been described and can be applied in certain circumstances, particularly with specimens from hospitalized patients and with autopsy specimens which are usually toxic and have low concentrations of viruses. (Part Four)

5. Determination of complement fixing (CF) antibody titres and immunoglobulins in venous and capillary blood samples showed higher titres with venous blood. The results of immunoglobulin determinations suggest that further careful study of this phenomenon should be undertaken since their implications are considerable in the context of paediatric diagnostic technique. (Part Five)

A P P E N D I X

APPENDIX

CITRATE - PHOSPHATE BUFFER

Stock Solution

A = 0.1M solution of citric acid (19.21 gm in 1000 ml)

B = 0.2M solution of dibasic sodium phosphate (28.39 gm of Na_2HPO_4
or 71.7 gm of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ in 1000 ml)

x ml of A + y ml of B.

x	y	pH
37.7	12.3	3.2
35.9	14.1	3.4
33.9	16.1	3.6
30.7	19.3	4.0
27.8	22.2	4.4
26.7	23.3	4.6
24.3	25.7	5.0
22.2	27.8	5.4
17.6	32.1	6.0
6.5	43.6	7.0

NB

pH 3.5 was obtained by making buffer solution of pH 3.4 according to above ratios and adjusting with the phosphate solution using pH meter. Similar procedure was followed in preparing buffers of pH 4.5, and 5.5.

PHOSPHATE BUFFER

Stock Solution

- A = 0.2M solution of monobasic sodium phosphate (31.2 gm of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ in 1000 ml)
- B = 0.2M solution of dibasic sodium phosphate (28.39 gm of Na_2HPO_4 or 71.7 gm of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ in 1000 ml)

A	B	pH
39.0	61.0	7.0
5.3	94.7	8.0

*SORENSEN'S PHOSPHATE BUFFER

Stock Solution

A = 0.1M solution of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (13.801 gm/litre)

B = 0.1M solution of Na_2HPO_4 (14.198 gm/litre)

x ml of A + y ml of B. Adjust pH with .1N NaOH and dilute with distilled water according to molarity required.

x	y	pH
2	48	7.92
0	50	8.98

* Modified from Histopathologic Technic and Practical Histochemistry (3rd edition) by R.D. Lillie, p. 664.

*METHOD OF TREATMENT OF SERA WITH PERIODATE
FOR REMOVAL OF NON-SPECIFIC INHIBITORS

M/90 Potassium periodate solution is prepared. Three volumes of the periodate are added to one volume of serum, mixed well and incubated for at least 15 minutes at room temperature. At the end of this time a volume of 1% glycerol-saline equal to that of the periodate is added to neutralize excess periodate.

(* W.H.O. Technical Report Series No. 170. Expert Committee on Respiratory Virus Disease (1959). p. 41.)

*TREATMENT OF SERA WITH CHOLERA FILTRATE
FOR REMOVAL OF NON-SPECIFIC INHIBITORS

One volume of serum plus four volumes of crude cholera filtrate are incubated overnight at 37°C; the mixture is then heated at 56°C for 1 hour in order to destroy cholera enzyme activity.

(*W.H.O. Technical Report Series No. 170. Expert Committee on Respiratory Virus Disease (1959). p. 37.)

R E F E R E N C E S

3

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