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Cytomegalovirus shedding and transmission
among children attending Halifax
day care centres
(A molecular epidemiological study)

by

Valerie M. Mann

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
at
Dalhousie University
Halifax, Nova Scotia
1989



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To the memories of my parents and Gilles, with love

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ABSTRACT

The enrollment of children in day care centres (DCC) is increasing rapidly. Infectious agents, such as cytomegalovirus (CMV), transmitted by close personal contact may be spread among the congregated children. DCC children from middle socioeconomic families have been shown to have higher CMV prevalences when compared to children cared for at home. No data were available for CMV prevalence and transmission in Canadian day care centre populations.

A survey of children attending Halifax, Nova Scotia day care centres was undertaken to determine the CMV excretion prevalence, to compare the CMV prevalence with reported day care centre population excretion prevalences and to the CMV seroprevalence in Halifax children surveyed approximately 20 years prior, and to determine whether CMV transmission had occurred.

Urine excretion of CMV by children attending five Halifax day care centres was used to determine CMV prevalence. Transmission data were obtained by repeatedly surveying the children attending one day care centre. Demographic information was collected by survey form.

Ten children from 75 children attending the five day care centres excreted CMV during a point survey. No risk factors could be identified for acquisition of CMV. The CMV excretion prevalence did not vary greatly from the seroprevalence found in the general Halifax children population 20 years prior. The CMV transmission data obtained from the repeated surveying of the population attending one day care centre yielded 13 of the 72 children participating excreting CMV, for a total of 20 CMV isolates. Two groupings of viral isolates were identified by restriction endonuclease analysis; one virus strain was excreted by two children during the first survey and the second strain was isolated from two children during separate surveys indicating the occurrence of CMV transmission among the day care center children. CMV transmission among children attending Halifax day care centres was found to be a rare event.

ABBREVIATIONS AND SYMBOLS

AIDS	Acquired Immunodeficiency Syndrome
°C	degrees Celsius
CF	census family
CF I	census family income
CID	cytomegalic inclusion disease
cm	centimeter
CMV	cytomegalovirus
CO ₂	carbon dioxide
cpm	counts per minute
CPE	cytopathic effect
D	many differences
DC	day care
DCC	day care centre
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
EDM	Eagles diploid medium
EDTA	ethylenediaminetetraacetic acid
F	1-2 band different
FCS	fetal calf serum
FCS:CS	fetal calf serum:calf serum
FDC	family day care
GM	growth media
GRS	garamycin reagent solution
g	gram

x g	relative centrifugal force
[³ H]	tritium
HC	home care
HNFF	human neonatal foreskin fibroblasts
HSV	herpes simplex virus
HSV-1	herpes simplex type 1
HSV-2	herpes simplex type 2
IDC	infant development center
I	3-4 band different
kb	kilobase
KW	Kruskal-Wallis Statistic
l	litre
L	long
M	molar
m	many
mg	milligram
ml	millilitre
mM	millimolar
MM	maintenance media
mm	millimeter
mo	months
mmol	millimole
MW	Mann-Whitney Test
NaCl	sodium chloride
ND	no differences
ng	nanogram

nm	nanometer
no.	number
ns	statistically not significant
P	private
P#	passage number
PBS	phosphate buffered saline
R	registered
rpm	revolutions per minute
S	short
ss	statistically significant
SDS	sodium dodecyl sulfate
STD	sexually transmitted disease
TCA	trichloroacetic acid
TE	tris-EDTA
TEA	tris-EDTA-acetate
TPE	tris-phosphate-EDTA
TRIS	tris (hydroxymethyl) aminomethane
TS	combined number of day care center spaces
uCi	microCurie
ug	microgram
ul	microlitre
UV	ultraviolet
V	volts
VZV	varicella-zoster virus
X	times
yr	years

number

? could not differentiate

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I. INTRODUCTION

Day care centre participation is increasing rapidly in our society. During 1984 approximately one-quarter of all children under the age of six years in the Halifax area, Nova Scotia, Canada, attended licenced day care centres (data supplied by the Nova Scotia Department of Social Services, Day Care Division). Cytomegalovirus (CMV), transmitted by close personal contact, may spread among the closely congregated children. Studies by Pass et al (1982, 1984), Hutto et al (1985A, 1985B) and Adler et al (1985A) have confirmed that children from "middle socioeconomic families", i.e. families in which at least one of the parents has a professional occupation (Pass et al 1982), attending day care centres in the United States have higher frequencies of CMV infection (as determined by CMV excretion) when compared with children from families belonging to the same socioeconomic status not exposed to that environment. Previously, children from middle socioeconomic families have been shown to have a lower CMV prevalence as compared to children from low socioeconomic families. Prior to the use of day care centres, early acquisition of CMV had been attributed to crowding and hygienic problems associated with low socioeconomic conditions (Pass et al 1984). The crowded environment

associated with low socioeconomic status is emulated in day care centres. Studies have indicated the basis for the spread of CMV within children attending the day care centres to be related to contact with CMV-contaminated surfaces, such as toys mouthed by CMV-excreting children (Pass et al 1982).

Concern regarding the frequent transmission of CMV among day care centre children was further emphasized by Adler (1988) after identifying 40 children excreting the same strain of CMV. At least 23 of the children were thought to have been infected in the day care centre environment during the study period. Although CMV infections in children attending day care centres are predominantly asymptomatic, the CMV-excreting child may transmit the virus to a susceptible seronegative female with child-bearing potential, for example the child's mother or a day care centre employee. More serious consequences may develop if the woman is pregnant. Possible consequences of a CMV infection during pregnancy include a congenitally infected newborn with sequelae such as microencephalopathy, congenital heart disease, and mental and motor retardation.

Literature on CMV within day care centre populations comprises studies from southern United States and Europe. No data are available for CMV prevalence (that is the relative number of children with CMV infections during a

specified period of time), and transmission in Canadian day care centre populations. The primary aims of this study were;

- a - to determine the CMV urine excretion prevalence and incidence for various Halifax day care centre populations.
- b - to assess whether risk factors could be identified for the excretion of CMV within the study populations.
- c - to ascertain whether common strains of CMV could be identified within the study population.
- d - to determine whether transmission of CMV could occur within the day care centre population.

Briefly, children attending several Halifax day care centres were surveyed for presence of CMV in excreted urine. Of the five day care centres, one centre was repeatedly sampled to determine whether children previously found not to be excreting the virus were excreting CMV. Restriction endonuclease analysis of the virus genomes was used to determine whether the virus was previously present in the population of children. If restriction endonuclease analysis of a virus isolate from a different child showed identity to a previously present virus, then transmission of CMV was concluded to have occurred among the children.

Information regarding various family demographic factors for the children was collected on survey forms distributed to the child's household.

This introduction consists of five sections; (A) a brief historical chronicle of CMV isolation, (B) a description of CMV epidemiology and clinical manifestations, (C) a review of the literature concerning CMV prevalence and transmission within day care centre populations, (D) a discussion of the basis for restriction endonuclease analysis of CMV genomes and (E) a description of the approach employed to determine whether CMV is present in Halifax day care centre populations and whether the virus is transmitted among the children attending day care centres.

A. HISTORICAL CHRONICLE OF CYTOMEGALOVIRUS ISOLATION AND
GENERAL DESCRIPTION OF THE VIRUS

In the early 1900's pathologists described unusual swollen inclusion-bearing cells found during the histological examination of the organs from children thought to have died from congenital syphilis (reviewed in Goodpasture and Talbot 1921). The organism believed responsible for the abnormal cells was the free living amoeba "*Endamoeba mortinatalium*". Goodpasture and Talbot disputed that these "protozoan-like" cells were actually protozoan in nature by proposing that the swollen inclusion-bearing cells were the effects of a viral infection. The associated cellular enlargement was termed "cytomegalia" (Goodpasture and Talbot 1921).

Cytomegalovirus [as later named by Weller et al (1960)] was first recovered from clinical isolates in the late 1950's by three research groups: Smith (1956), Rowe et al (1956), and Weller et al (1957). All three groups recognized the species-specific aspect of CMV infection, (that is the inability of the virus to infect other species), noting the failure of virus isolated from humans to infect a different species (e.g. mouse and rabbit).

A description of the virus produced effects on a cell

culture was included in Smith's (1956) report on CMV isolation from clinical specimens. Clinical CMV isolates obtained from submaxillary salivary glands and kidney tissue from infants under three years of age (one who died from congenital inclusion disease [CID]) were propagated in human cells. The tissues were ground, then inoculated into cell culture tubes containing human uterine wall fibroblast cells. The description of the cytopathic changes to the cells in culture was as follows; "small, round or oval foci in which the cells were enlarged, rounded or oval, and somewhat refractile in contrast to the normal fibroblasts...There was a continuous slow increase in the number and size of the lesions in all tubes, followed by degeneration of cells in the centre of the lesion. The degenerating central cells became granular and disintegrated, leaving masses of dense refractile granules". Virus-antibody neutralization tests using sera from an infant with CID and from the infant's mother confirmed that the viruses were of human origin (Smith 1956).

An epidemiological survey for the presence of CMV antibodies in serum samples was performed by Rowe et al (1956) using CMV isolated from adenoids of asymptomatic children undergoing tonsillectomy-adenoidectomies. Three different isolates producing intranuclear inclusion bodies, including the now designated prototype strain AD169, were recovered. The serological prevalence of complement-fixing

antibodies to CMV AD169 was evaluated for a survey sample which included a wide age spectrum, ranging from newborn to over the age of 35 years. The data revealed a rise in the prevalence of complement-fixing antibodies to AD169 from the age of six months (Figure 1). Infants sampled at birth via cord bloods were resampled at the age of six weeks to determine whether the antibodies initially detected were maternally acquired: i.e. transferred to the fetus via the placenta. A decline in the antibody titre would occur as the maternal antibodies were cleared from the serum. In all cases the titre was lower at six weeks than at birth, indicating the presence of maternal antibodies to AD169.

A similar virus was isolated from children manifesting a syndrome representing CID by use of roller tube cultures of human tissue (Weller et al 1957). Nonhuman cell cultures were also examined for potential use in isolating virus; however, the cells did not support growth of virus. Three isolates of CMV were recovered, including the now-called Davis strain isolated from liver biopsy material. Other isolates from urine were also obtained. Weller et al (1957) deduced that because cytomegalic cells were excreted in urine, possibly virus was also excreted, and proposed that the presence of virus in urine may be of diagnostic value.

The viruses isolated by Smith (1956), Rowe et al (1956) and Weller et al (1957) were initially termed "Human

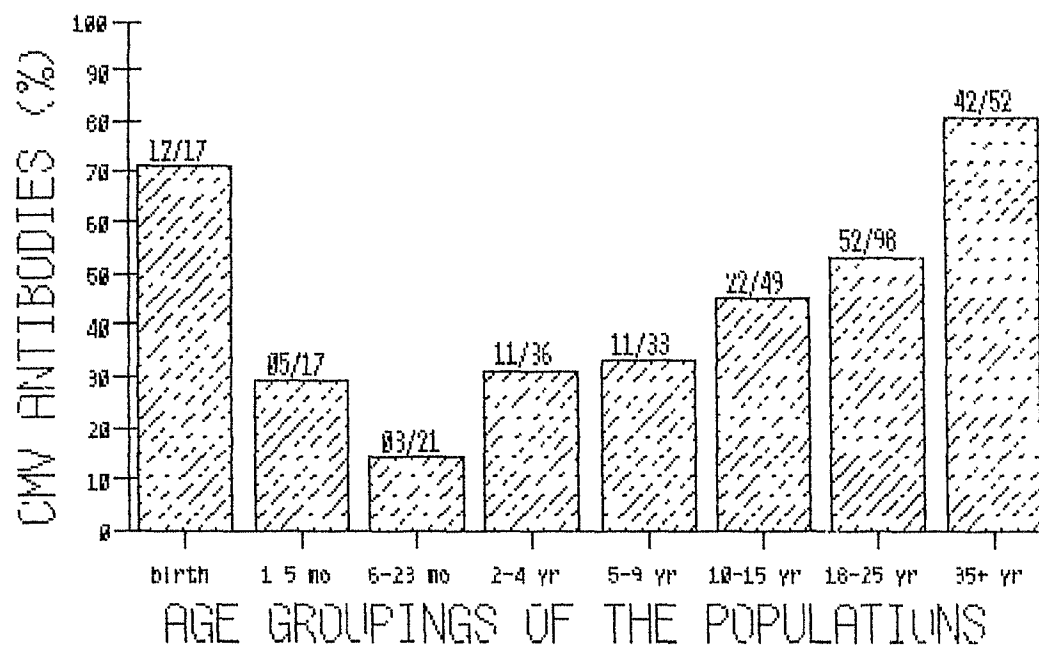


FIGURE 1 Percentage of individuals containing complement fixing antibodies to AD169 in different age groups (Rowe et al 1956). Data given in the paper adapted graphically.

Salivary Gland Virus" or "cytomegalic inclusion disease virus". In 1960 Weller *et al* proposed the name Cytomegalovirus for both the viruses isolated from human and animals. The manifestations of the disease caused by the human virus ranged from asymptomatic in the children undergoing tonsillectomies to death of infants with congenital cytomegalic inclusion disease.

CMV belongs to the Herpesviridae family of viruses. These viruses range in size from 100 to 350 nm and consist of an icosahedral capsid, containing double-stranded DNA enclosed within an envelope derived from the host cell's nuclear membrane. Disease manifestations of human infections caused by these viruses vary from localized cold sores [Herpes Simplex Virus type 1 (HSV-1)] to systemic infectious mononucleosis [Epstein-Barr Virus (EBV)]. A common characteristic of the members of this virus group is the ability to become latent within the host.

Latency has been defined as "the presence of the viral genetic information in an unexpressed state in the host cell" (Lamberson 1985). The viral manifestations may reoccur when the proper stimulus is provided. According to Lamberson (1985) CMV fulfills the latency definition since CMV post-perfusion syndrome can occur after blood transfusion with blood from which the virus could not be isolated. The site of latency for CMV is not known;

however, salivary tissue, heart tissue, prostate tissue, splenic B lymphocytes, and monocytes have been proposed as possible sites (Jordan 1983). A persistent form of virus infection also occurs, in fact, CMV has been shown to be excreted for months or years after the initial infection (Stagno et al 1975).

CMV *in vivo* infects primarily epithelial cells; however, *in vitro* the virus can only be easily propagated in fibroblastic cell culture (Rowe et al 1956). Smith (1986) disputes the claim that CMV only infects fibroblastic cells *in vitro* suggesting that other parameters including ploidy and extent of differentiation influence the susceptibility of a cell to CMV infection. Due to the cell-associated nature of the reproductive cycle of CMV, the virus is not readily recovered from the cell culture medium. In contrast *in vitro* HSV cell infection has easily recoverable virus contained in the cell culture medium.

The Herpesviridae family contains three subfamilies (Fraenkel-Conrat 1985);

Alpha-herpesvirinae [HSV-1, Herpes Simplex Virus type 2 (HSV-2), varicella-zoster (VZV)]: the members of this viral group have been shown to have a variable host range, a short replication cycle, a highly progressive CPE in tissue, and to remain latent in the neural ganglia.

Beta-herpesvirinae (CMV): CMV is the only member of this group which infects humans. The members of this viral group are characterized by a narrow host range, a relatively long replication cycle, a less dramatic CPE than seen with the alpha-herpesvirinae. Latency may occur.

Gamma-herpesvirinae (EBV): EBV is the only member of this group which infects humans. These viruses exhibit a narrow host range, infect specifically either B or T-lymphocytes (with either a lytic, latent or persistent infection mode), and have a reproductive cycle which varies in length and ensuing cytopathology.

Members of the CMV group of viruses have been isolated from many animal species including humans, old world and new world simians, and equines, felines, porcines, murine and other rodent species (Fraenkel-Conrat 1985). Only weak cross-hybridization is found between the genomes of human and monkey CMV (Hayward et al 1984). Human and simian CMV can be further differentiated into strains on the basis of restriction endonuclease analysis of the viral genome (Huang et al 1980A & B).

B. EPIDEMIOLOGY AND CLINICAL MANIFESTATIONS OF HUMAN CYTOMEGALOVIRUS INFECTION

In order to discuss CMV infections in children attending day care centres, an understanding of CMV infections and their epidemiology is required. This section provides an overview of the epidemiology and clinical manifestations of human CMV infections.

1. MODES OF CYTOMEGALOVIRUS TRANSMISSION

Several modes of transmission occur with CMV infections. Close or direct personal contact seems to be common to all forms of CMV transmission (Table 1).

a. ORAL SECRETION

Infectious CMV has been detected in human oral secretions (Pass et al 1982, Hutto et al 1986, Schupfer et al 1986). Pass et al (1982) proposed that CMV transmission via surfaces contaminated by oral secretions containing CMV was at least partially accountable for the spread of this infection within day care centre populations. Saliva containing CMV placed on surfaces outside the body was found by Schupfer et al (1986) to remain infectious for time periods up to 2 hours, although Hutto et al (1986) could

TABLE 1 Reported modes of CMV transmission.

Close personal contact has been associated with the many modes of CMV transmission. The virus has been isolated from many body fluids. This table represents a compilation of all the reported modes of CMV transmission.

<u>SOURCE</u>	<u>VEHICLE</u>	<u>PORTAL OF ENTRY</u>	<u>REFERENCE</u>
a. ORAL SECRETION	HANDS, KISSING, SHARING OF SALIVA CONTAMINATED OBJECTS	MOUTH	PASS ET AL 1982
b. URINE EXCRETION	HANDS	MOUTH	WELLER ET AL 1957
c. GENITAL SECRETION	SEXUAL CONTACT PARTURITION	GENITAL MUCOSA MUCOUS MEMBRANES	HANDSFIELD ET AL 1985 STARR 1979
d. BREAST SECRETION	INFANT FEEDING	MOUTH	AHLFORS & IVARSSON 1985
e. BLOOD TRANSFER	TRANSFUSION TRANSPLACENTAL	BLOOD FETAL BLOOD	EMBIL ET AL 1968 STARR 1979
f. ORGAN TRANSPLANTATION	DONATED ORGAN	SURGICAL WOUND	CHOU 1987

only recover infectious CMV for 30 minutes after a surface was contaminated by saliva containing CMV. According to Hutto et al (1985B) sampling saliva for virus is not as accurate as the use of urine samples for determining presence of virus. When children attending day care centres were tested for excretion of CMV both in urine and saliva, saliva virus isolation was found to be less efficient than from urine.

b. URINE EXCRETION

A common manifestation of CMV infection is persistent CMV excretion for extended periods of time, for example years in urine. Within this time frame the virus may be shed only intermittently as shown by both Weller et al (1957) and Faix (1985). Transmission of the virus via urine is possible since urine excreted virus has been found to be infectious. Infectious CMV from urine-contaminated diapers could be recovered for time periods of 2 hours from the absorptive surface and for time periods of 8 hours from a nonabsorbent surface (Faix 1985).

c. GENITAL SECRETION

Two modes of CMV transmission are associated with genital secretion of the virus: sexual contact and parturition. CMV has been isolated both from semen and the

female genital tract secretions (Embil et al 1985A, Handsfield et al 1985, M=Farlane and Koment 1986); however, there are other vehicles of transmission, i.e. saliva and skin, which may be factors in sexual transmission of the virus. Transmission of CMV between sexual partners was documented in a study of patients seeking treatment at a Sexually Transmitted Diseases (STD) clinic by Handsfield et al (1985). CMV was found to be present in urine and cervical secretions from women and, in some cases, semen and urine specimens from their partners. Isolated viruses from the sexual partners were analyzed by restriction endonuclease digestion of the viral genome. This technique identifies possible epidemiologically relationship between the viruses, that is whether one CMV-infected partner transmitted the virus to the other partner. (This technique is discussed in detail in Section D.) Two pairs of regular sexual partners had identical strains of CMV virus, in the cervical secretions and the man's urine indicating transmission of the virus between the partners. The third pair of sexual partners were determined to have epidemiologically unrelated viral strains, thus transmission of the virus had not occurred between these partners.

Transmission of CMV from a husband to his wife was documented by junctional hybridization (methodology discussed later), as well as clinically and serologically by Demmler et al (1986). A previously CMV-seronegative

pediatric house officer found to have a primary CMV infection, excreted virus in his saliva, urine and semen. Approximately five months later his previously seronegative wife began excreting CMV in her saliva and vaginal secretions. The viruses isolated from the husband and his wife were analyzed and found to be identical strains of CMV; thus indicating that the wife had contracted her infection from her husband. It could not be determined, however, whether the route of transmission was oral or genital because of the nature of their relationship.

In longitudinal studies both single and multiple CMV infections have been documented. Promiscuous women (prostitutes addicted to heroin) were repeatedly tested for the presence of CMV infection (Wertheim et al 1985). Five patients from this longitudinal study had multiple CMV isolates over periods of four weeks to 15 months, depending on the individual studied. Restriction endonuclease analysis of the virus isolates from each patient determined that for each patient only one virus strain was shed over the longitudinal time period. Each woman was infected with a single strain of CMV and probably exogenous reinfection had not occurred. Patients excreting multiple strains of CMV have also been identified.

Multiple CMV isolates also obtained from STD patients were analyzed by restriction endonuclease digestion by

Chandler et al (1987). Four patients were identified as excreting multiple strains of CMV virus, including one case in which the cervical virus isolates were obtained five months apart. CMV isolates obtained from another patient excreting CMV concurrently in urine and cervical secretions were ascertained to be different viral strains (Chandler et al 1987). McFarlane and Koment (1986) were also able to concurrently isolate two strains of CMV from a male patient: one strain isolated from urine and the second strain from semen.

Women with an active cervical CMV infection at the time of parturition are thought to spread the virus to the neonate during the birth process via an infected birth canal (Weller 1971A & B). The CMV present within the mother's genital tract may come in contact with the neonate during the passage through the birth canal, resulting in an acquired CMV infection (Pass 1985). CMV-infected semen as a possible route for congenital infection of the fetus was examined by Demmler et al (1986). An infant was conceived while the father was excreting CMV in his semen; however, no evidence of congenital CMV infection was found.

d. BREAST SECRETION

Another route of CMV transmission can be via breast milk. Infants nourished by breast milk containing CMV may

develop perinatal CMV infections. The presence of CMV in breast milk samples from 35 donors including both CMV seropositive and seronegative women was evaluated by Ahlfors and Ivarsson 1985), only the seropositive women excreted the virus in their milk. The time period in which the virus was first found to be secreted into the milk was approximately one to two weeks post-delivery and peaked after two to 12 weeks post-delivery. According to Pass, 30% of infants nursed by seropositive mothers will acquire CMV infections, and the prevalence increases to 70% when CMV could be isolated from the breast milk (Pass 1985).

CMV viral isolates obtained from two mother-infant pairs were analyzed by restriction endonuclease analysis (Garrett and Warren 1985). Samples consisted of CMV isolated from the mother's milk and the infant's urine. In both pairs of mothers and infants, restriction endonuclease analysis determined that the CMV isolates from each pair of breast milk and urine samples were identical indicating the probable transmission of the virus via the breast milk.

e. BLOOD TRANSFER

Transmission of CMV via blood has two modes: vertical - transplacental from mother to fetus, and horizontal - from person to person via blood transfusion. The exact route of infection for a fetus congenitally infected *in utero* by CMV

is not yet known; however, intrauterine infection is thought to occur via viremic spread of the virus through the transplacental blood supply (Alford et al 1981).

CMV infections may be also transmitted during transfusion of fresh blood into recipient patients. Numerous studies have described, in detail, the occurrence of CMV infections in patients following blood transfusions (Embil et al 1968, Adler 1983, Wilhelm et al 1986), even though CMV is not generally able to be isolated from the donor blood samples (Beneke et al 1984). Beneke et al (1984) have proposed that blood donors with active CMV infections, as determined by the presence of CMV specific IgM antibodies, transmit CMV infections through their blood. CMV seronegative blood has not been shown to transmit CMV infections (Pass 1985).

Sixteen pediatric child heart surgery patients were evaluated prior and post-extracorporeal circulation to determine the incidence of CMV infection through blood transfusions (Embil et al 1968). CMV infection was exhibited by seven children as determined by a post-operative rise in CMV specific complement-fixing antibodies. Five of the patients developed a post-perfusion syndrome and the other two patients remained symptom-free. In a further study by Wilhelm et al (1986) CMV seronegative patients receiving blood transfusions were assessed to

determine CMV seroconversion. Of the 592 seronegative patients participating in the survey, seven patients seroconverted with the presence of CMV specific IgM antibodies indicating a primary CMV infection. All seven patients who seroconverted had received blood containing CMV antibodies.

Maternally acquired antibodies to CMV are thought to provide some protection from the severe manifestations of a primary CMV infection to immunodeficient newborns. A newborn infant of a CMV seronegative mother transfused with blood from a CMV seropositive donor may present with severe manifestations; for example neonatal sepsis-like syndrome or death. The passive immunity conferred by maternal antibodies to CMV would not be present in such a newborn (Betts 1983).

f. ORGAN TRANSPLANTATION

Renal transplantation studies have shown that CMV infection is uncommon if both the kidney donor and recipient were CMV seronegative, and common if the donor was seropositive and the recipient seronegative (Borkowsky 1984). Viral isolates from four transplantation recipients receiving either a heart or kidney from a seropositive cadaver were analyzed by restriction endonuclease analysis (Chou 1987). Three of the four patients, one heart

recipient and two kidney recipients, seroconverted within six to 12 weeks post-transplantation. All four recipients subsequently shed the CMV strain isolated from the seropositive cadaver from which their organs were obtained. One previously seropositive heart recipient was found to shed the same strain of CMV as the cadaver, indicating a reinfection and not a reactivation of the previous infection.

Two kidney recipients, one seronegative for CMV and the other seropositive, receiving donor kidneys from the same CMV seropositive cadaver began to excrete CMV in both their urine and saliva after transplantation (Grundy et al 1986). The excreted viral isolates were identical when analyzed by restriction endonuclease digestion; therefore, the patients were infected by the virus present in the donor kidney.

Reactivation of previous CMV infections has also been documented in transplantation patients. CMV infections associated with bone marrow transplantation were studied by Winston et al (1985). Four patients shedding CMV asymptomatically prior to transplantation subsequently developed CMV infection manifestations. The viruses isolated prior and post-transplantation were analyzed by restriction endonuclease digestion and found to be identical indicating that the virus present prior to transplantation was responsible for the symptoms manifested after

transplantation.

Recommendations to avoid CMV infection during transplantation have been proposed. Sommerville (1984) concluded that the risk of a primary CMV infection in transplantation patients warrants the screening of the patients to determine their immune status to CMV. A seronegative patient should receive only CMV seronegative blood and transplants from seronegative donors (Onorato et al 1985).

2. DESCRIPTIVE EPIDEMIOLOGY OF CYTOMEGALOVIRUS INFECTIONS

Epidemiological surveys reporting CMV infections usually describe the number of cases of infection in terms of CMV prevalence. The prevalence is defined as the number of cases of a disease or infection within a specified period in a defined population (Halsey 1986). The CMV prevalence within a given population may be assessed by a variety of methods including serological survey and virus isolation. Each of these methods has both advantages and disadvantages associated with its use. Unfortunately the prevalences reported for each method cannot be directly compared against each other.

A serological survey of a population for the presence of CMV will identify persons with CMV antibodies. The

distinction between people currently infected with the virus and those individuals who have had an infection in the past is not usually made. Also, individuals excreting the virus at the time of the survey can not be identified, and therefore the virus is not isolated for further epidemiological analysis such as restriction endonuclease analysis.

Virus isolation will identify individuals infected and currently excreting CMV. CMV prevalence determined by urine excretion will identify only those individuals excreting CMV in their urine at the time of the survey. As a result individuals with CMV infections but not excreting the virus in their urine will not be identified. Also, individuals excreting virus in body fluids other than urine will not be identified as infected with CMV. The virus isolation method to determine CMV prevalence also allows for further epidemiological analysis to be performed such as restriction endonuclease analysis. This method has the further advantage that the procedure does not involve an invasive technique such as the venipuncture utilized in serological surveys.

CMV prevalence surveys can also be classified into two subdivisions; point prevalence and period prevalence. A point prevalence survey of day care centre children for the presence of CMV will involve a single sample from each child

in a relatively short period of time. A period prevalence may involve a number of samples from each child at different time points over a given period of time, possibly years. A point prevalence survey can only determine the prevalence; whereas, the period prevalence can identify both the prevalence and incidence of infection.

The epidemiology of a disease 's generally described in the terms of the parameters of time, place and person. Numbers of CMV infections have been found to remain relatively constant throughout the year not varying seasonally (Murph et al 1986, Adler 1985A). CMV infection is ubiquitous: the distribution is worldwide. Numerous studies involving CMV antibody prevalence have determined that the major factor responsible for the geographic variation of CMV infections of healthy individuals is the socioeconomic factor and, hence, the crowding index (persons/room) of the sample population (Sarov et al 1983). In young populations CMV antibody prevalence is the highest in underdeveloped countries such as Tanzania and St. Lucia, and lowest in the developed nations in Europe and North America (Ho 1982A, Gold and Nankervis 1983) (Figure 2). Gold and Nankervis (1983) noted that a pattern exists in the aquisition of antibodies by age for most populations. There is a loss of the transplacentally acquired CMV antibodies during the first year of life; after which, an increase in antibody prevalence during the young adult

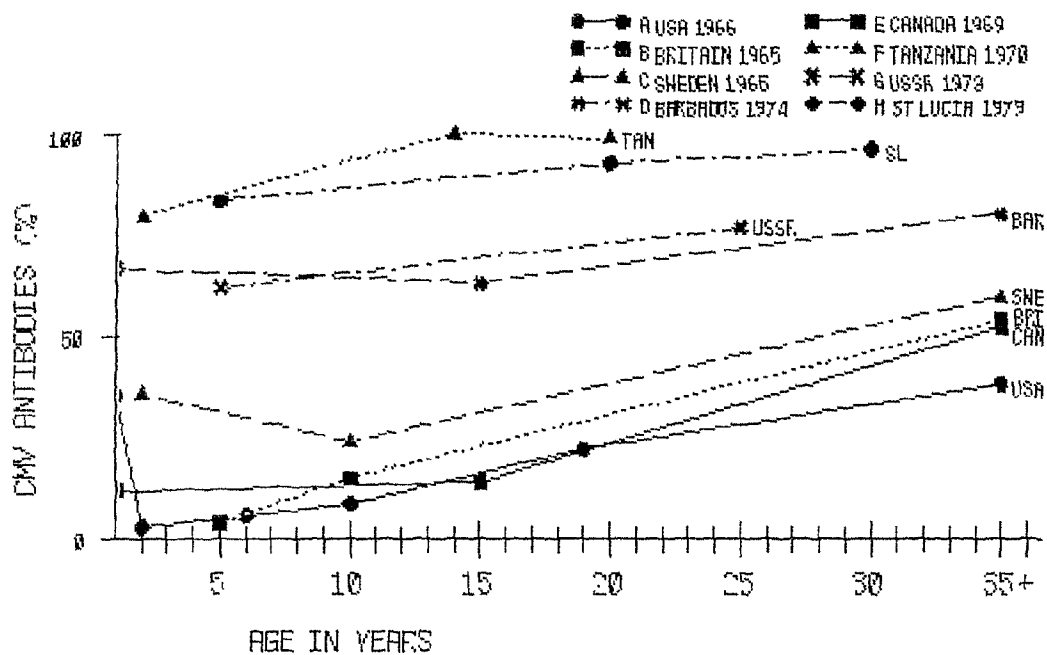


FIGURE 2 CMV serological prevalence in general populations from various geographic locations. Adapted graphically from selected data provided (Gold and Nankervis 1983).

ages occurs followed by a levelling off. The age of acquisition of CMV depends on the socioeconomic status and race of the individual (Evans 1983).

Populations can be categorized by age, race, socioeconomic status, sexual habits, occupation, and immune status. All of these elements may influence the prevalence of CMV within a given population. Race and socioeconomic factors are considered to be indicators of overcrowding. The close personal contact found within an overcrowded population may lead to virus transmission among that population (Griffiths et al 1985). Surveys for antibody levels to CMV (Embil et al 1969A, Pass et al 1982, Ahlfors 1984, Nelson et al 1987) indicate the individual's sex does not influence the prevalence of CMV infection at any given age; however, Betts (1983) disagrees. Occupation has also been shown not to be a factor in CMV transmission (Alford et al 1981, Stagno et al 1986A,).

a. AGE

The age at which a person may become infected by CMV has been found to be related to many factors including geographic location, socioeconomic status, and personal behaviour practices (Lamberson 1985). These factors reflect the crowding index of the population (Sarav et al 1983). A general description of the prevalence of CMV infections

based on compilations of serological studies on United States populations has indicated that 10 to 30% of children have been infected by the age of ten years. The CMV antibody prevalence rises to 20 to 50% for women of child-bearing age, and by the age of 90 years or more 60 to 90% of all individuals have evidence of prior CMV infection (Lamberson 1985).

The prevalence of CMV infection for a given age and population has been found to be lower in Canadian populations than in United States populations (Embil et al 1969A). The CMV seroprevalence in healthy Halifax populations was assessed during the period from 1967 to 1968 by presence of CMV antibodies. Included in the survey were cord blood samples from newly delivered infants, blood samples from preschool aged children attending "well baby" clinics, children aged 5 to 17 years of age, and adult blood volunteers from the Red Cross, encompassing a broad socioeconomic cross-section of the community. An overall CMV seroprevalence of 22.4% was found, with a general rise of percent positive from birth to above 50% for the population 40 years and over. The number of positive samples for CMV antibodies significantly rise at 2 to 3 years of age and in the twenties. The rise in number of people with CMV antibodies occurs at the ages when there is an increase in social contact.

A similar serological survey was effected in urban Swedish populations (Ahlfors 1984). The CMV seroprevalence rose approximately 10% each decade after birth: a consistent increase in antibody levels with age. Although the large rises in CMV prevalence occurring at 2 to 3 years and in the early twenties found by Embil et al (1969A) were not reflected in Swedish populations, the overall trend of rise in CMV seropositivity with age was consistent. The rise in CMV seroprevalence was also reflected in Houston, Texas, USA children evaluated for presence of CMV antibody in blood samples from birth until 10 years of age (Yow et al 1987). In this population the largest acquisition of CMV antibodies occurred from birth to two years and again at 5 to 6 years.

Sarov et al (1983) evaluated healthy Israeli children from three different populations: children cared for at kibbutzim, children from urban areas and Bedouin children (Figure 3). The major difference between these groups of children was the age at which close contact with many other children occurred. The kibbutz children were cared for by communal day care-like centres from an early age, in "high standards of hygiene and good socioeconomic conditions". The urban children attended nursery school at approximately 2 to 5 years of age, which was their first encounter with large groups of children. The Bedouin children began school at approximately 6 to 9 years of age until which time they did not have contact with large numbers of children. The



FIGURE 3 CMV antibody frequency in different populations of Israeli children. Increases in CMV antibody frequency were found to occur when the children were first exposed to large groups of other children. Adapted graphically from data given in the paper (Sarav et al 1983).

CMV seropositivity rise within each group reflected the age of initial contact with large numbers of other children. The highest CMV seropositivity was found in the kibbutz children. Their seropositivity was already high in the first two years of life and increased at 2 to 5 years. The urban children displayed an increase in their CMV seropositivity at the age when attendance in nursery school occurred. The Bedouin children exhibited an increase in the CMV seropositivity at the ages of the first exposure to school. The CMV seropositivity was found to be correlated with crowding of the children.

Acquisition of CMV within the family unit was assessed by Taber et al (1985). Once one member of a family was infected with CMV, a high level of intrafamilial transmission of CMV within the family setting occurred. Because there is increased CMV infection in young children (Embil et al 1969A, Ahlfors 1984), a seronegative mother in families with young children would be at risk to acquire the virus.

b. RACE

Race has been described to be a determinant in CMV infection (Betts 1983). A United States population cross-section of different races aged 20 years were surveyed for the presence of CMV antibodies. From the results of the

survey, Blacks were more likely than Caucasians to have evidence of CMV infection by the age of 20 years (Betts 1983). Griffiths et al (1988) similarly surveyed another United States population of 1000 pregnant females for presence of CMV antibodies. Noncaucasian women had higher prevalences of CMV antibodies when compared, controlled for age, to Caucasian women. Caucasian women acquired CMV antibodies at the same rate as the noncaucasian women; however, the initial rates were lower. Female Asian and Blacks were ascertained to have comparable CMV antibody prevalences. In 1988 Griffiths et al evaluated 1989 middle to upper class pregnant women. Again race and educational status (reflecting the socioeconomic status) were the strongest predictors of CMV seropositivity.

c. SOCIOECONOMIC STATUS

Atlanta hospital patients, representing low, middle, and upper incomes classes were assessed for serological presence of CMV infection (Public Health Reports 1967). The details of the different socioeconomic division were not described. The low income group consistently had high prevalences of CMV antibodies at an earlier age and also had higher serological CMV positivity for all age groups than the other income classes. Comparison of women of child-bearing age determined the CMV seropositivity to be influenced by the women's socioeconomic status. The

seropositivity was found to increase as the socioeconomic status of the population decreased. Griffiths et al (1985) attributes the higher CMV prevalences in the low socioeconomic status classes to overcrowding and close personal contact. The survey of people aged 20 years by Betts (1983) also exhibited higher prevalence of CMV antibodies in the lower socioeconomic populations.

Cabau et al's (1979) evaluation of populations from Paris, France for CMV antibodies also demonstrated that CMV seropositivity during the early infancy age period was influenced by socioeconomic class. The socioeconomic divisions were based on the family's home location and education. Low and middle socioeconomic status mothers and their children attending two public health centres were examined to determine serological evidence of CMV infection when the child was 10 months of age, and again at two years of age. The low socioeconomic mothers' CMV seropositivity was found to be higher than that of the middle socioeconomic mothers. The infants from the low socioeconomic conditions also exhibited a higher CMV seropositivity as compared to the middle socioeconomic class. The increase in CMV seropositivity within the population was attributed to differences in breast feeding patterns and socioeconomic status. Cabau et al (1979) attributed the socioeconomic status as the most important factor in the CMV seropositivity during the first 2 years of life, and in this

study the mother was identified as the child's main CMV infection source. It should be noted that restriction endonuclease analysis evidence was not used to support the conclusions.

d. SEXUAL HABITS

The importance of CMV as a sexually transmitted disease in heterosexual populations was questioned by Knox (1983). Other researchers, such as Handsfield et al (1985) and Demmler et al (1986), have demonstrated the effect of sexual practices on the transmission of CMV infection. Four different populations attending clinics in Halifax, Nova Scotia, Canada were assessed for CMV presence (Embil et al 1985A). Three clinics included clientele with routine gynecological prenatal care; the fourth clinic was an STD clinic. The population attending the STD clinic had the highest prevalences of CMV antibodies suggesting a possible sexual route of transmission.

Sexual partners of the patients attending an STD clinic were assessed to evaluate the transfer of CMV to sexual partners using restriction endonuclease analysis of the isolated viruses (Handsfield et al 1985). Other nonsexual modes of transmission, for example routine personal contact, were not considered. The sexual partners of CMV seropositive patients had a higher CMV seropositivity as

compared with the sexual partners of CMV seronegative patients. Partners of women shedding CMV exhibited a higher prevalence of viral shedding. Restriction endonuclease analysis of viral isolates from two pairs of sexual partners showed identical viral strains in each pair. The third pair were infected with different strains of CMV, that is epidemiologically unrelated viruses.

Indirect epidemiological relationships may also be identified by use of restriction endonuclease analysis of CMV isolated from a population. Identical virus strains were isolated from urine samples from a male and female patient attending a STD clinic claiming no sexual relationship (Chandler et al 1987). Further analysis determined that the two patients had, in fact, sexual relationships with a common male partner, indicating a sexual route of transmission. A cluster of identical virus isolates was also identified being comprised of one sexual pair of patients, the above mentioned non-sexually related pair and a patient from an obstetric clinic. Chandler et al (1987) proposed that many indirect epidemiological relationships existed within the observed population.

The high seroprevalence found in homosexual men has been associated with the large number of sexual partners found within this lifestyle (Kryger et al 1984). Mindel and Sutherland (1984) compared homosexual, bisexual and

heterosexual men for CMV antibodies. The highest CMV antibody levels were found in the homosexual male population. Lange et al (1984) also evaluated healthy asymptomatic homosexual men for prevalence of CMV. All 30 subjects evaluated had CMV-specific antibodies; whereas, only 23% of the control group of heterosexual men had CMV antibodies. When this group was examined for virus excretion, 11 subjects were shedding the virus in either semen, urine, or both.

Although CMV prevalence has been associated with sexual habits, Pass (1985) pointed out that "it would be a mistake, however, to refer to CMV as a sexually transmitted disease; acquired infection rarely results in disease and, since virus is shed in saliva as well as from genital secretions, oral contact may be as important as genital contact. On a worldwide basis it is very likely that most persons acquire CMV before sexual maturity".

3. CYTOMEGALOVIRUS DISEASE MANIFESTATIONS

Previously CMV infections were only thought to be responsible for the severe manifestations associated with congenital CID; however, researchers now recognize CMV to be one of the most common virus infections (Hayes 1985). CMV infections present with a wide variety of manifestations, the severity of the symptoms dependent upon the

immunocompetence of the individual. CMV is an important etiologic agent of congenital infections, a significant cause of transfusion acquired infections, and a factor in morbidity among organ transplantation recipients and other immunocompromised patients. Infected immunocompetent individuals, such as otherwise healthy young adults, may remain asymptomatic. However, in an immunocompromised patient, the symptoms of infection may be disseminated and life threatening. Recently the role of CMV in oncogenesis has been questioned and several studies are currently trying to determine whether an association exists (Spector and Spector 1984, El Beik et al 1986).

a. CONGENITAL INFECTION

Congenital CMV infection is defined by the isolation of CMV from an infant within the first two weeks of life. The isolation of CMV from an infant during this period is considered evidence for an *in utero* infection resulting from the transplacental transmission of the virus (Pass 1985). From 0.5% to 2% of all infants born in Canada and the United States are congenitally infected with CMV; however, less than 5% of the congenitally infected infants have clinical manifestations (Embil et al 1969A, Starr 1979). Surveys of Canadian infants have found a 0.55% and a 0.42% congenital CMV urine excretion prevalence (Embil et al 1969A, Larke et al 1980). Of asymptomatic congenitally infected infants, 10

to 15% exhibit developmental abnormalities within the first ten years of life (reviewed in Starr 1979).

Congenital infection manifestations range from asymptomatic to severe life-threatening symptoms including intrauterine growth retardation, jaundice, hepatosplenomegaly, chorioretinitis and rarely ascites. Congenital deformities may include clubfoot, indirect inguinal hernia, high arch palate and congenital heart disease. Neurological sequelae may consist of mental and motor retardation, spasticity, seizure disorders, impaired vision and hearing loss (reviewed by Starr 1979, Larke et al 1980). A poor prognosis exists for neonates with severe early manifestations.

Only primary CMV infections of a seronegative child-bearing female were believed, in some cases, to result in congenital infections with manifestations. This was based on the observation that congenitally infected infants were usually the first born of a woman (Weller 1971A & B). Seropositive women have delivered congenitally infected infants resulting from a reactivation of maternal infection (Preece et al 1986). Nankervis et al (1984) proposed that an active infection during the latter stages of pregnancy is associated with an increased risk of congenital infection. Although CMV specific antibodies from a previous CMV infection will not protect the fetus from congenital CMV

infection, the manifestations from such an infection will likely be less severe (Stagno and Whitley 1986B).

b. ACQUIRED INFECTION

NEONATES:

As many as 20% of neonates may become infected with CMV during passage through a CMV-infected birth canal. Approximately 3 to 12 weeks after birth these infected children begin to excrete CMV in their urine. However, the manifestation tends to be an asymptomatic infection (reviewed by Starr 1979). A neonate may also become infected with CMV via blood transfusion (Betts 1983). Clinical manifestations of an acquired CMV infection may include splenomegaly, hepatomegaly, elevated liver enzymes, thrombocytopenia and pneumonitis (Starr 1979).

INFANTS AND CHILDREN:

CMV infection in infants and children is usually subclinical; however, mild abnormalities may be seen including hepatomegaly, splenomegaly, spider angiomas, respiratory symptoms, pneumonitis and fever (reviewed by Starr 1979, Betts 1983). A heterophil negative mononucleosis with exudative tonsillitis has been reported in children (Pannuti et al 1985).

ADULTS:

Adults infected with CMV are usually asymptomatic. A heterophile negative mononucleosis may be seen in young adults consisting of fever, lethargy, malaise, splenomegaly, arthralgias and lymphadenopathy (reviewed by Starr 1979, Betts 1983). The mononucleosis may result from a primary infection or infection with a different CMV strain. If the CMV infection was transmitted via a blood transfusion, symptoms of this self-limiting infection are similar to those seen with mononucleosis (Starr 1979, Adler 1983). CMV has also been reported in patients with idiopathic inflammatory bowel disease (Berk et al 1985).

IMMUNOCOMPROMISED PATIENTS:

CMV is the most important herpes virus infection in immunocompromised hosts (Skinhoj 1985). Severe immunodeficient infants present with disseminated CMV infections, along with other opportunistic infections. In acute leukemia patients infected with CMV, fever, pneumonitis, chorioretinitis or mononucleosis-like symptoms may occur. The CMV infection may disseminate to the lungs, spleen, liver, adrenal glands, myocardium and gastrointestinal tract (reviewed by Starr 1979).

Within a year post-transplantation, 60 to 90% of allograft transplantation and bone marrow transfusion patients excrete CMV. Infection may occur via two modes: reactivation of a latent CMV infection or spread of the virus present in the infected organ, marrow or blood (Chou 1987). Clinical manifestations in the transplant patient are more likely to occur with a primary infection rather than with a reactivated infection (Borkowsky 1984). Recurrent CMV infection may be as serious, with the same symptoms as the primary infection; however, the onset is insidious (Sommerville 1984). Manifestations of infection include fever, pneumonia, hepatitis, leukopenia, atypical lymphocytosis, chorioretinitis and encephalitis onset one to four months after transplantation (reviewed by Starr 1979). Bone marrow transplantation patients with CMV infection most commonly present with interstitial pneumonia, approximately two months post transplantation (Betts 1983). Some acquired immunodeficiency syndrome (AIDS) patients have been found to have CMV pneumonia and follow the general symptoms of other immunocompromised patients (Lamberson 1985, Drew et al 1984).

C. CYTOMEGALOVIRUS INFECTION IN DAY CARE CENTRE POPULATIONS

In Canada the rapid growth in number of children attending day care centres is evident in the increase in the number of day care centre spaces registered with the National Day Care Centre Information Centre. Included in the number of day care centre spaces are family day care spaces. Family day care is defined as registered private families who give care to children during the day. In 1984, the number of children in Canada attending day care centres or family day care homes was 171,654: almost double the 1980 figure of 109,141 and approximately three times the figure of 55,181 for ten years earlier in 1974 (Health and Welfare Canada 1987). In fact from 1973, when the National Day Care Information Centre first published data regarding Canadian day care centres, to 1986 the most recent survey, the number of full-time day care centre spaces has increased eightfold (Health and Welfare Canada 1987) (Figure 4). Within the expanded use of day care centres, the largest age group in attendance is comprised of children three years of age, followed by children aged four years old; whereas, the infant group comprises the largest number of spaces in family day care (Figure 5) (Health and Welfare Canada 1987).

Day care centres in Nova Scotia have been found to exhibit the increases found for the total number of Canadian day care centre spaces. The number of day care centre

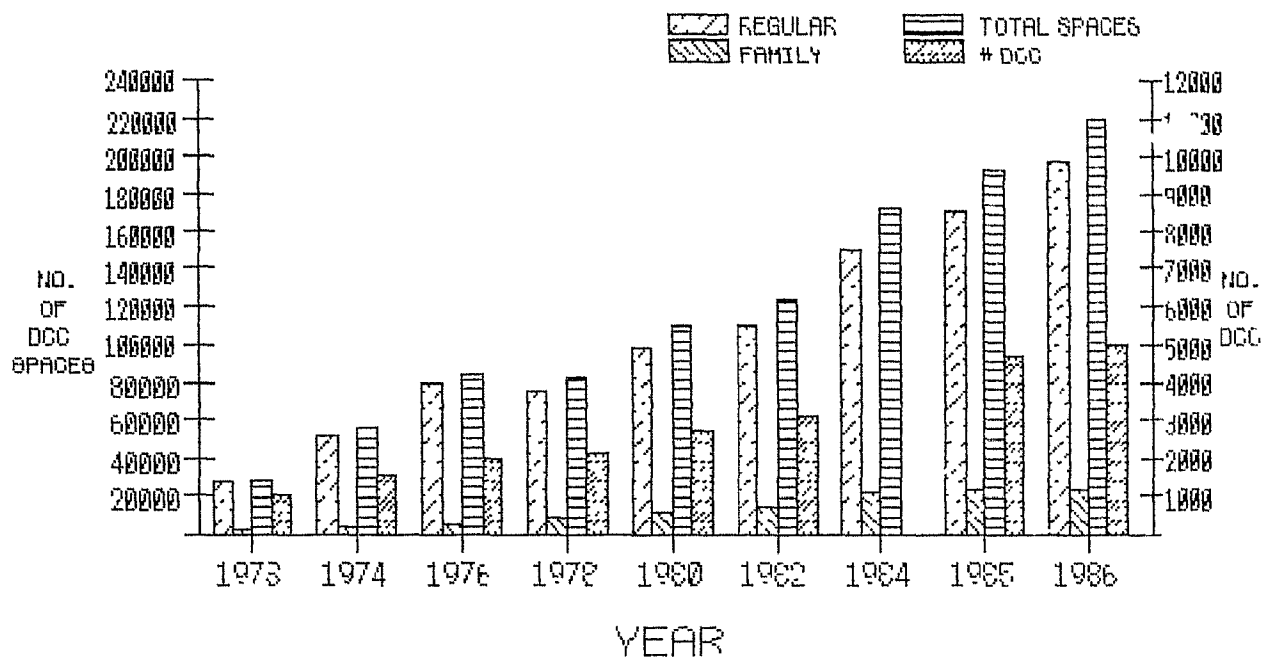


FIGURE 4 Canadian day care centre spaces and number of day care centres by year from 1973 to 1986. The number of day care centre spaces are categorized according to whether the spaces are regular day care centre spaces (REGULAR) or family day care spaces (FAMILY). The combined number of spaces is represented by TOTAL. The number of day care centres (# DCC) for each year is also presented. Data adapted graphically (Health and Welfare 1987).

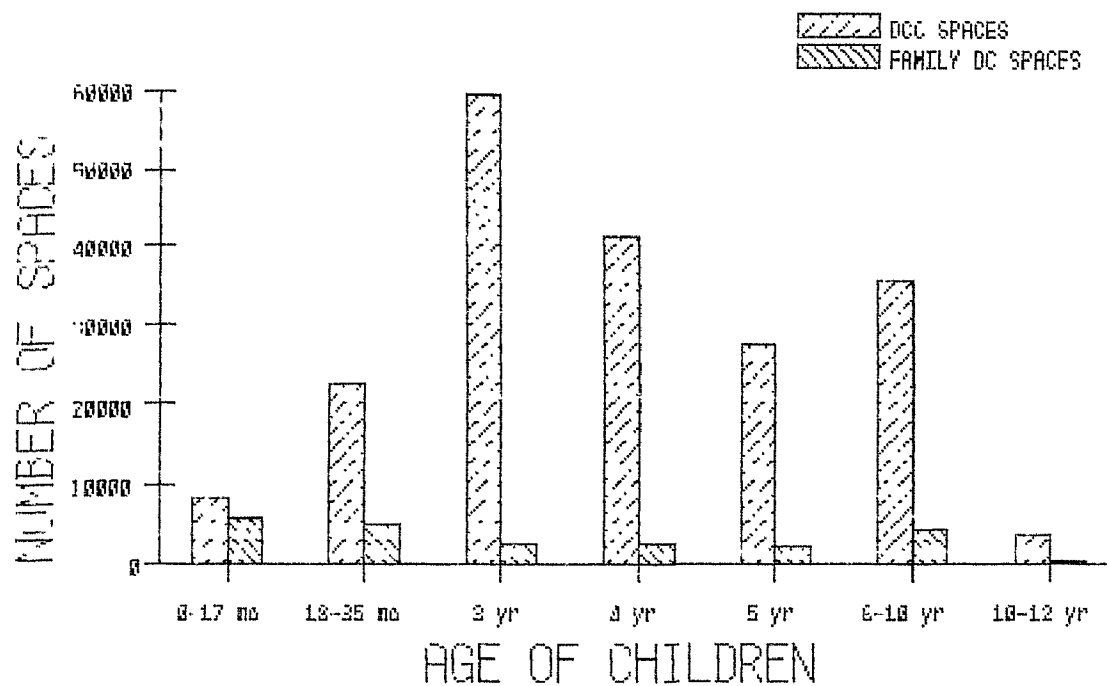


FIGURE 5 Day care spaces classified by age for day care centre spaces (DCC SPACES) and family home care spaces (F DC Spaces). Data adapted graphically from the National Day Care Study (Health and Welfare Canada 1987).

spaces in Nova Scotia has almost doubled from 2,675 in 1980 to 4,865 in 1986, approximating the rate of increase in the total number of Canadian day care centre spaces for the same time period (Health and Welfare Canada 1982, 1987). In the Halifax area there were 6,790 children under the age of six years (data from the 1981 Halifax census, Statistics Canada 1982). According to the Department of Social Services, Day Care Division, 1,921 children under the age of six years were enrolled in day care centres in the Halifax area in 1984 (personal communication, Nova Scotia Department of Social Services, 1984). Given that the number of Canadian children is not increasing rapidly - there has, in fact, been a decline in the number of children aged up to nine years from 1976 to 1981 (Statistics Canada 1986)- it can be seen that approximately one-quarter of all Halifax children under the age of six years attend licenced day care centres. It has been emphasized already that when children are kept in an environment which allows prolonged close personal contact, infectious agents such as CMV, transferred by close personal contact, may be transmitted among the children.

The increased use of day care centres as a means to care for children has been attributed to the expanding numbers of mothers leaving the home environment to join the labour force. Two factors accounting for women leaving the home environment to join the work force have been defined. The first factor is the mother being the sole provider for

The first factor is the mother being the sole provider for the family, and the second factor is the rising cost of living expanding beyond the limits of one salary (Health and Welfare Canada 1978). According to the Canadian Minister responsible for the Status of Women, the Honourable Barbara McDougall (Health and Welfare Canada 1987) only one Canadian family in six conforms to the description of the traditional family with an employed, sole-supporting father and housewife mother. Resulting from the increase in women joining the working force is the increasing number of Canadian children under the age of six years whose mothers participate in the labour force. For example in 1978 there were 695,000 Canadian children under the age of six years with working mothers. By 1986 the number had doubled to 1,210,108 with the current trend in the labour force towards an increasing participation by women (Statistics Canada 1982).

When Canada's population growth over the decade 1971 to 1981 was compared against other western nations, such as the United States, Britain and France, Canada exhibited the largest population growth within the given time period (Statistics Canada 1986). Although the Canadian population had increased in number, alterations within the structure of the population occurred. The percentage of women in the childbearing years of 20 to 39 has risen, but the birth rate declined (Statistics Canada 1986). The number of children

under the age of 14 also decreased; however, the numbers of children attending day care centres expanded. The responsibility for the increased use of day care centres may be influenced by another aspect of the population exhibiting alterations; the rapidly enlarging Canadian divorced population. Partly as a result of this altered population, the number of single parent families in Canada also expanded (Statistics Canada 1982). Society has been affected by the rising number of single mother families through the increased demand for day care services.

In the United States similar employment trends for women are exhibited, approximately one half of women with children under the age of six years are now wage earners (Haskins and Kotch 1986). Currently approximately 7.8 million United States children under six years of age have mothers in the labour force and of those, 4.7 million receive care outside the home. The projected figures are 10.5 million and 6.3 million, respectively, for the year 1990. In the United States, of these children whose mothers work, 40% are cared for by family relatives either at the child's home or outside of the child's home. Day care centre attendance accounts for 18% of the children and the remaining children are in family day care: that is, a non-relative cares for the child in the child's own home (Zigler and Muenchow 1986).

1. VIRUS ISOLATION FROM DAY CARE CENTRE CHILDREN & PARENTS

Day care centres have been a popular resource for child care in Swedish populations. An undesirable consequence has been respiratory infections in the children attending these day care centres. The presence of many pathogens, including CMV, was evaluated by Strangert et al (1976). The six month study included 51 children in attendance at a Stockholm day care centre, as well as, 40 children cared for at home. The children ranged in age from six months to four years, usually being from single-parent families living in smaller dwellings. Antibodies to CMV were present in seven of the day care centre children and in four of the children fostered by home care. The children excreting CMV did not show any signs of infection; therefore, Strangert et al (1976) noted that asymptomatic CMV infections were common among young children in Sweden.

In the same year, two groups of children attending a suburban Stockholm day care centre were also assessed by urine and saliva samples for the presence of CMV (Strangert et al 1976). The first group ranged in age from seven to 20 months and the second group ranged in age from 21 to 30 months. No child from the first group (children aged seven to 20 months) was found to be excreting CMV. Overall 35% of children excreted CMV. Seven out of ten children, children aged from 21 to 30 months, were found to be positive for the

presence of CMV. To exclude the possibility of transmission of an isolated infection inflating the CMV excretion prevalence, a second group of 40 children, aged six months to three years, attending thirteen different day care centres were evaluated for CMV excretion. Nine out of the 40 symptom-free children were found to be CMV excretors. The placement of CMV-excreting children in quarantine was not recommended because asymptomatic CMV infections were frequent among young Swedish children (Strangert et al 1976).

The viral genomes of the CMV isolates obtained in the 1976 survey of children attending Stockholm day care centres (Strangert et al 1976) were analyzed by restriction endonuclease digestion to determine whether CMV was transmitted among the children within the day care centre environment (Grillner and Strangert 1986). Three CMV isolates obtained from children attending one day care centre were found to have identical electrophoretic fragment patterns by restriction endonuclease analysis indicating transmission of the CMV from one of the children to the others. The remaining four isolates had different patterns, representing epidemiologically distinct viruses. Interestingly, one of the day care centres included a set of twins, both of which excreted CMV. A different strain of CMV (different restriction fragment patterns) was found to be excreted by each of the twins, indicative of a CMV

infection source other than the mother for at least one of the twins. The identity of the mother being the potential CMV source was based on speculation, no family member of the twins was examined for CMV excretion. The viruses isolated from children attending a different day care centre were all unique, as were the remaining day care centre virus isolates.

Two Swedish day care centre populations were surveyed over a ten month period to determine CMV excretion prevalence (Grillner and Strangert 1988). Six out of 16 children and eight out of 15 children were excreting CMV. Restriction endonuclease analysis of the DNA from the CMV isolates did not find evidence of day care centre CMV transmission among the children (Grillner and Strangert 1988).

Pass et al (1982) were the first researchers to evaluate middle to upper socioeconomic day care centre populations in the United States for the presence of CMV. The children attending the Alabama day care centre and participating in the survey were predominantly Caucasian with approximately equal numbers of girls and boys, ranging in age from three to 65 months. The children's parents were well-educated and mostly professionals. CMV was excreted by 36 out of the 68 children, with 13 out of 29 children excreting the virus in saliva. Overall 30 out of 70

children excreted CMV in urine and/or saliva. The highest excretion was seen in children aged from 13 to 24 months and the lowest was seen in the infant group, children aged up to 12 months. Pass suggested that if CMV-antibody blood screening techniques were used to evaluate the children attending the day care centre, the CMV prevalence would be approximately 10 to 30% higher. Urine culture for CMV presence only identifies children currently excreting the virus. In contrast, the antibody test identifies all children who are currently excreting virus or have had a previous CMV infection.

CMV infection within the day care centre population was higher than expected for middle to upper socioeconomic status children within that age group. In fact, the frequencies of CMV infection displayed approached the high levels reported for developing nations. This high CMV excretion prevalence could not be attributed to lower socioeconomic conditions since the families involved in the study belonged to middle to upper socioeconomic classes and should have low CMV infection levels. The high CMV prevalence was hypothesized to be due to the increased opportunity for transmission of CMV among the day care centre children due to the close contact amongst children, and the airborne spread of virus through aerosols or transfer of virus by the hands of employees. The issue of whether the children excreting CMV could also be a source of

possible infection for seronegative women of child-bearing age and potential with possible consequences of a congenitally infected infant was raised (Pass et al 1982).

A comparison of the prevalences of CMV infection between children cared for at home and the children attending the above mentioned day care centre confirmed the day care centre environment influenced the transmission of CMV in preschool aged children (Pass et al 1984). The day care centre children were evaluated from September 1981 to October 1982. The control group of children consisted of patients from approximately the same age range attending an Alabama pediatric office practice within the same location as the day care centre. Because of the location of the practice, the control population was assumed to reflect the demography of the families of the day care centre children. Sick children were excluded from the study. The demographic information obtained revealed some similarities between the two groups. The statistically significant differences found were the higher number of siblings for the home care children and a higher percentage of the day care centre parents were divorced. Urine samples from 70 out of a possible total of 75 day care centre children were evaluated by cell culture techniques for CMV excretion. Repeat specimens were collected from some of the children. Overall CMV urine excretion was found in 59 out of 103 day care centre children. Pass concluded that the majority of

children in this centre acquired CMV infections between 12 and 18 months of age. This is the age range at which they have transferred from an infant class to a toddler class. These children have become ambulatory resulting in close personal contact with the other children attending the day care centre. This result is similar to the previously published report by the same group for day care centre children described earlier (Pass et al 1982). In the control children CMV prevalence was examined by obtaining urine samples, of which five out of 47 were positive for CMV excretion. Blood samples were also evaluated for serological presence of CMV antibodies in the control children: 18 out of 109 children exhibited antibodies to CMV. The prevalences for serological evidence and excretion of CMV in the home care children were within the previously reported range (Pass et al 1982); that is, the day care centre children's infection levels were higher than the excretion prevalence exhibited by the control children thereby implicating the day care centre in the transmission of CMV within the populations of preschool aged children. Because of the possibility that a day care centre child could be a vector in horizontal transmission of CMV to his or her seronegative child bearing mother, the mothers of the day care centre children were also assessed for presence of CMV antibodies. Twenty of the 50 mothers were seronegative for CMV antibodies. Of the mother-child pairs studied, 11 of the 50 day care centre pairs consisted of a seronegative

mother with a child excreting CMV, whereas within the home care control mothers, only 1 of the 72 mothers with a CMV infected child was seronegative (Pass et al 1984).

The higher CMV excretion prevalence in the children attending the day care centre as compared to the prevalence (both serological and excretion) in control children prompted the evaluation of three day care centres serving middle to upper income families to determine if the previous results had been biased by an unusually high CMV excretion prevalence within one particular day care centre (Figure 6) (Hutto et al 1985A). The previous day care centre was reassessed along with other new day care centres and a control population of 66 children and mothers from the patient clientele of a suburban physician (as in the previous 1984 study). The day care centre populations differed only slightly, with DCC 2 containing greatest proportion of noncaucasian children. The three day care centres had similar percentages of children excreting CMV with the highest infectivity being exhibited by the 25 to 36 month old children. For comparative purposes with the control children, the authors combined the data collected from the three individual day care centres. Of the 188 day care centre children evaluated for the presence of CMV excretion, 77 children were found to excrete the virus. When the 66 home care control children were assessed for CMV antibodies only 10 out of 66 children were seropositive and

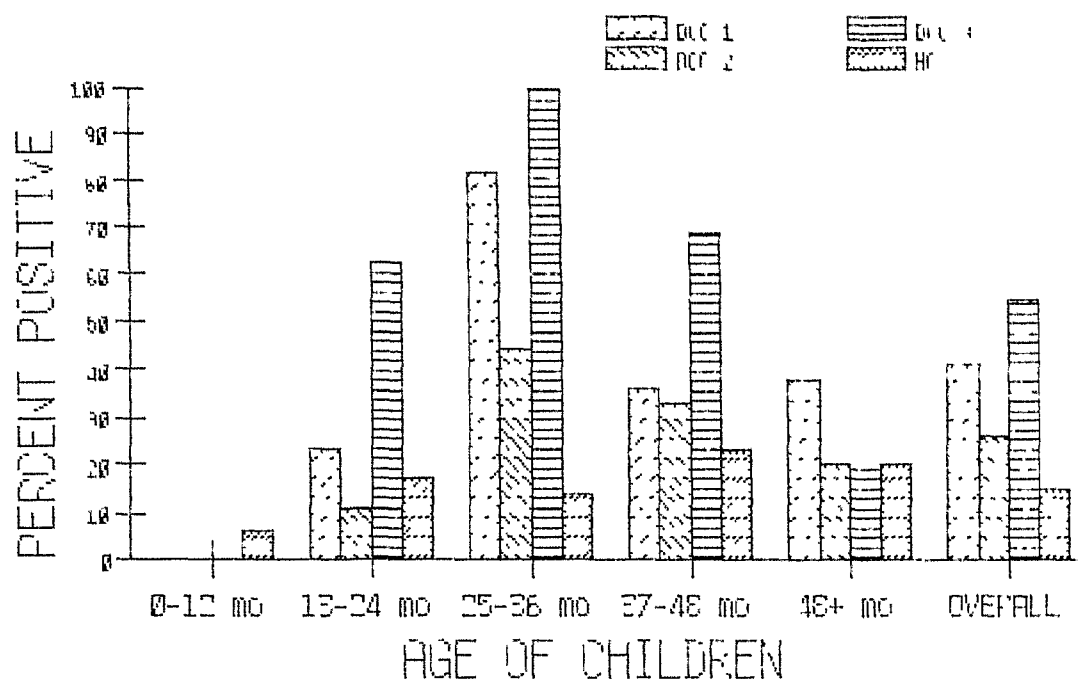


FIGURE 6 Urine CMV excretion prevalence in children attending day care centres (DCC 1,2 & 3) compared with children cared for at home (HC). The overall CMV excretion prevalence (OVERALL) is also compared for the four groups of children. Data adapted graphically (Hutto et al 1985A).

only 2 out of 25 children excreted CMV. Apart from the day care children's number of siblings and higher percentage of divorced parents, the only other factor which differed between the two groups was that the day care children were in close personal contact with other children for approximately 40 hours a week. The higher CMV excretion prevalence supported their original results and the conclusion that the CMV prevalence was higher in children attending a day care centre.

The CMV isolates from children in an Alabama day care centre were analyzed with restriction endonucleases (Hutto and Pass 1984). [This abstract did not identify from which day care centre the CMV isolates had been obtained. Therefore, it is not possible to determine whether these isolates were from the original centre or whether they were from one of the two additional centres included in the 1985 study (Hutto et al 1985A).] Restriction endonuclease analysis of an unmentioned number of CMV genomes revealed two groups of CMV strains. The first strain was from three children, aged 22 to 26 months, grouped in the same classroom. The second group of CMV isolates with identical restriction endonuclease patterns were from three other children, aged 28 to 34 months. The fact that several children were excreting CMV with identical restriction endonuclease patterns suggests that CMV had been transmitted horizontally among the children at the day care centre.

During the 24 month study period, two out of 35 susceptible mothers of day care centre children seroconverted, possibly due to a child excreting CMV. However, no restriction endonuclease studies were reported to confirm whether the viruses from the infected mothers were the viruses previously present in their children.

Further analyses were performed on the data obtained during the 1985 study (Hutto et al 1985A) from children attending three day care centres and children cared for at home, and by survey of the children's parents both by questionnaire and serologically (Pass et al 1986). The only statistically significant difference discovered was the number of female parents participating in the study. Thus, the parent(s) of the home care group consisted of mothers of the control children; whereas, the parents of the day care children included both mothers and fathers. This difference could be accounted for by the fact that the home care children were brought to the physician's office by their mothers. The parents of the day care centre children seroconverted more frequently, 14 out of 67 day care centre children parents as compared to 0 out of 31 home care children's parents. No correlation between seroconversion and sex, race, age, number of years educated, marital status, occupation or number of children at home could be found. Parental excretion of CMV was found to be consistent with having a CMV-excreting child attending a day care

centre. There were two pregnancies among mothers who seroconverted. In the first case the neonate appeared normal, however, no viral isolation attempts were made. In the second case the neonate appeared normal, but when first evaluated for CMV excretion at the age of four months, the infant was discovered to be excreting CMV. Viral transmission was assumed to be via breast milk. The excretion of CMV by a child was linked to the seroconversion in the parents. [Adler (1986D) noted this study failed to establish the day care centre as the source of virus by restriction analysis of the virus isolates, although confirmation by restriction endonuclease analysis was performed at a later date (Pass et al 1987).]

CMV isolates from seven families with recent maternal or congenital infection were analyzed by restriction endonuclease digestion and gel electrophoresis of the DNA isolated from the viruses (Pass et al 1987). The study groups consisted of five patients confirmed to seroconvert or who had given birth to a newborn infant with a congenital CMV infection. The remaining two patient groups were nonpregnant mothers with children in the day care centre from Pass et al's original studies (1982, 1984). Restriction endonuclease analysis showed that the viruses isolated from each family group consisted of an identical strain (five newborn, sibling and mother-groups and two mother-child pairs attending the day care centre) but all

seven viral strains were different from one another. In the case of the two day care centre children's CMV viral isolates, restriction endonuclease analysis showed transmission between the children and their mothers. The chronology of the CMV infections of the mother-child pairs supported the conclusion that the child had transmitted the virus to its mother, as both of the day care centre children began excreting virus prior to their mother's seroconversion. These results further confirmed Pass et al's concern that the mothers of day care centre children could be at risk to acquire CMV from their children (Pass et al 1982).

Five Alabama day care centre populations, three middle income and two low income centres, were assessed during March to June 1984 by a single urine and saliva collection to obtain an approximation of the CMV excretion prevalence within the day care centre populations (Figure 7) (Hutto et al 1985B). Serum samples from the day care centre staffs and the parents of the children, were also included in the survey. Overall CMV excretion prevalences for the children varied from 9% to 49%. Surprisingly, the frequencies of virus excretion in both urine and saliva specimens from children attending the lower socioeconomic centres were lower than those found in middle to upper day care centre populations. No explanation was proposed to account for this. The age of the children differed between the two

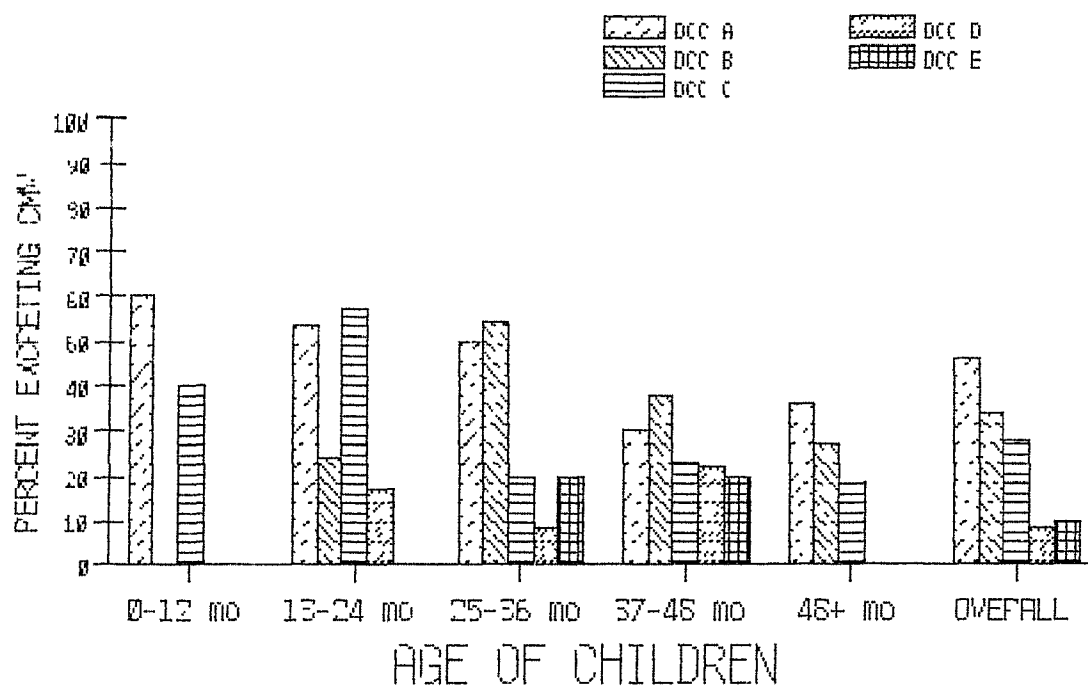


FIGURE 7 Urine CMV excretion by children of various ages attending five Alabama day care centres (DCC). OVERALL represents the overall CMV excretion prevalence in each day care centre. Data adapted graphically (Hutto et al 1985B).

populations, children attending the low socioeconomic day care centres were older than the children attending the middle socioeconomic centres. Age of the children attending the lower socioeconomic centres was found to average approximately 40 months; whereas, the age of the children attending the middle socioeconomic centres averaged approximately 34 months. It is possible that the low socioeconomic children had had CMV infections at an earlier age and had ceased shedding virus in their urine. As previously demonstrated, this study found CMV frequencies higher than expected for children of that age range. Early studies showed that the frequencies of CMV in United States preschool aged children ranged from 10% to 20% (Lamberson 1985). The higher frequencies were hypothesized to result from contact with numerous children found in a day care centre environment.

Other day care centre populations have also been surveyed to determine the CMV prevalences. The population from a Virginian day care centre situated on a hospital campus was surveyed three times (Adler 1985A). The study population consisted of 66 children aged from three months to five years and one child eight years of age. The demographic features of the population were not discussed with the exception that the parents of the children were hospital staff. CMV excretion was exhibited in 16 out of 66 children. The CMV excretors were found to be older, mean

age 3.4 years old, as compared to the mean age of the non CMV excretors, 2.4 years of age. When restriction endonuclease *EcoRI* was used for analysis of the 16 day care centre virus isolates, two groups of virus isolates were identified: one consisting of seven isolates from children less than 29 months and the other comprised of four from children older than 36 months. The remaining five isolates were unique. This grouping of viral isolates corresponded approximately to the room arrangements of the children, evidence that at least nine children were infected with CMV at the day care centre. Five children, enrolled at the day care centre during the study, began excreting CMV. Three of these isolates were unique suggesting outside transmission. However, internal transmission also occurred as indicated by two children excreting CMV strains with restriction endonuclease patterns already present within the day care centre. Controls for this study consisted of 531 aged matched hospitalized children; 36 children were determined to be excreting CMV.

The earlier results regarding the occurrence of viral transmission from day care centre children to their parents were confirmed in a second study (Adler 1986A & C). The parents of the day care centre children were assessed for presence of previous CMV infection by CMV antibody; nine out of ten mothers of CMV excreting children were seropositive for CMV, 12 out of 33 mothers of non-CMV excreting children

were seropositive, and 133 out of 393 control women were seropositive (not matched for socioeconomic status, age or whether they had children in day care). When the CMV isolates from the parents of the day care centre children were studied by restriction endonuclease digestion analysis the isolates from three parents, one father and two pregnant mothers, were identical to the CMV isolated from their children. A conclusion that CMV was transmitted from child to parent was reached.

Further confirmation of CMV transmission from the day care centre children to their parents and caretakers has been found (Adler 1988). A 26 month survey consisting of 104 children from a day care centre, as well as, their families found three predominant CMV strains. Of the children, 26 children acquired strain A, three children acquired strain B, five children became infected with strain C and four children began to excrete unique CMV strains. Most of the children infected with CMV during the study were under the age of three years. Although many children had become infected with CMV, Adler (1988) concluded that CMV transmission among the day care centre children is slow to occur.

Children and staff from a middle to upper socioeconomic day care centre in Iowa City, Iowa were evaluated for the presence of CMV excretion during a fall and spring survey

(Figure 8) (Murph et al 1986). Of the children attending the day care centre, 41 of the 48 children were assessed during one six month interval for CMV excretion in both urine and saliva. From the fall survey 8 out of 39 children were found to excrete CMV, 9 out of 41 children excreted CMV during the spring survey. The highest excretion prevalences were displayed by the toddler group, agreeing with the previous studies by Pass et al (1982, 1984), and Jones et al (1985). The CMV excretion within the day care centre children was found to be stable regardless of the season the survey was conducted. The demographic features of the parents only differed significantly in the mother's education (by use of statistical analysis - test not named). Three strains of virus, A (four isolates), B (three isolates) and C (one isolate) were identified by restriction analysis. The presence and transmission of CMV within the day care centre was attributed to poor hygienic condition (although the claim was not substantiated) and virtually unlimited mobility of the toddlers.

The natural history of CMV infections in children attending day care centres was investigated by Murph and Bale (1989). The CMV acquisition rate in a large group day care centre was ascertained to be 12.6% per year. Most children begin to excrete CMV in their second year of day care centre attendance. Children excreting CMV were found to be younger than the nonexcretors. The quantity of virus

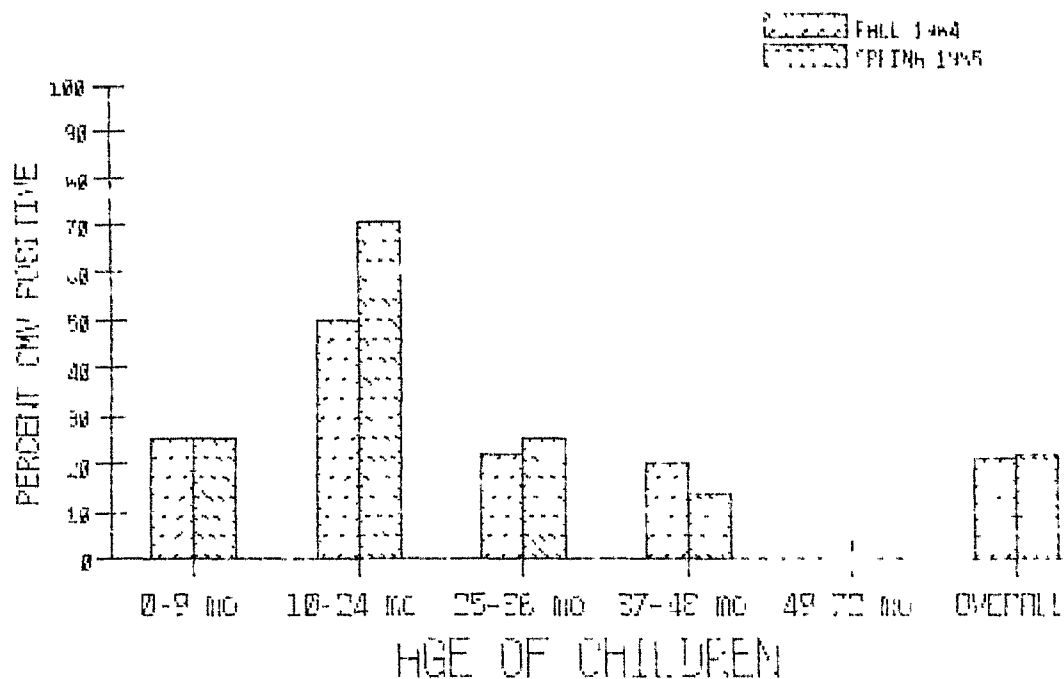


FIGURE 8 CMV urine excretion prevalence in children attending day care centres during different seasons. A day care centre was surveyed during the fall of 1984 and spring of 1985. Data adapted graphically (Murph et al 1986).

excreted during the initial infection was found to be comparable to the quantities of virus excreted by congenitally infected children. However, the day care centre children's virus excretion declined rapidly in comparison to the congenitally infected children.

Concern regarding the possibility of whether developmentally delayed children pose a higher risk for CMV transmission prompted the evaluation of children attending both for infant developmentally delayed centres (IDC) and regular day care centres in the San Francisco Bay area to determine whether a difference in CMV excretion prevalence between the two groups existed (Jones et al 1985). A total of 11 day care centres was assessed: seven infant developmentally delayed centres and four regular day care centres of various socioeconomic classes. The overall prevalence of CMV excretion was similar for both the regular day care centres and the developmentally delayed centres. Of the children attending day care centres, 20 out of 90 children excreted CMV and 11 out of 50 children from the developmentally delayed centres were found to excrete CMV. In both types of centres, and as with Pass et al (1982, 1984) and Murph et al (1986), the highest CMV frequencies were exhibited by the toddler groups. Jones et al (1985) concluded that no justification existed for excluding developmentally delayed children from therapeutic and educational programs on the basis of CMV excretion.

Different types of day care centres were also examined for the presence of CMV excreting children in England (Nelson et al 1987). Five day care centre nursery populations were assessed over a one year period; three ordinary health care centres and two nurseries for the physically, emotionally or intellectually handicapped children. Overall 32 out of 117 children excreted CMV, the lowest numbers of excretors were found in the older and handicapped children. The special nursery centres exhibited low numbers of children excreting CMV, in fact one centre had no evidence of CMV presence. The day care centre children were found to have CMV excretion prevalences similar to those found by surveys in United States day care centre children populations (Hutto et al 1985B). Twenty out of 32 viruses isolated from the day care centres were analyzed by restriction endonuclease analysis; four groups of viruses with identical restriction endonuclease patterns were identified. Nurseries A and C each contained contained siblings with identical isolates and Nursery B contained two groups of isolates: one group consisted of virus isolates from siblings and the other group consisted of isolates for two unrelated children. The remaining strains were unique. Problems in propagating the virus were responsible for the inability to analyze the remaining 12 virus isolates. The authors concluded that the restriction endonuclease analysis supported the conclusion that most of the children had

obtained the virus from outside the day care centre in spite of two centres containing congenitally infected children.

The influence of day care centre attendance on the transmission of CMV among young children has been disputed by Yow et al (1987). A longitudinal serological survey for presence of CMV antibodies (from birth to ten years of age) was conducted using children from Houston, United States. The CMV seroprevalence for children attending day care centres was determined to be 36%, as opposed to 26% for children cared for at home. Statistical analysis found the CMV prevalences to be not significantly different between the two groups of children. The children surveyed were not restricted to middle to high socioeconomic families (as were the children studied by Pass et al (1982, 1984) but encompassed all socioeconomic classes. This may have biased the results, the children from the higher socioeconomic families would be more likely to be attending day care centres which would cause a higher CMV infection; whereas, the children from lower socioeconomic families would have a higher CMV prevalence as compared to the higher socioeconomic non day care centre children as a result of exposure to other factors postulated to be related to increased CMV prevalence.

2. DAY CARE CENTRE ENVIRONMENTAL STUDIES

Studies on the excretion prevalence and the patterns of transmission of CMV in day care centres suggest that the day care centre environment, for example toys, countertops, etc., may be important in the transmission of the virus among the children (Pass et al 1982, 1984, 1986, Hutto et al 1985A, Jones et al 1985, Murph et al 1986, Grillner and Strangert 1986). In Pass et al's (1982) initial study on CMV prevalence in day care centre children, surfaces of toys were swabbed to determine whether CMV virus could be isolated from a toy recently mouthed by a CMV-excreting child and thus, be a source of transmission for the virus. Four plastic toys, after being mouthed by day care centre children, were determined to have CMV on the surface; however, no survival time for the virus was determined.

Although Faix (1985) was not directly studying CMV survival in day care centres, his evaluation of CMV survival on environmental surfaces to determine the role of fomites in the CMV transmission in nurseries is relevant to the question of CMV transmission in the day care centre environment. Surfaces in direct contact with CMV-infected secretions around infants in an intensive care nursery were found to be contaminated by CMV. Aliquots of urine samples obtained from the CMV-excreting infants were inoculated onto disinfected Plexiglass surfaces and also cotton bedding.

Swabs from the surfaces were cultured from one to 48 hours post-inoculation. Infectious virus could be recovered eight hours after inoculation from the nonabsorbent surface, and up to two hours from the absorptive surface. Survival time also varied with the amount of virus in the inoculum.

In a similar study to determine the survival of CMV, experimentally inoculated saliva and urine soaked paper diapers at a concentration similar to that found in CMV-excreting children were evaluated for presence of infectious CMV (Schupfer et al 1986). Saliva containing CMV was infectious for up to 2 hours either at room temperature or 37°C, depending on the dryness of the sample. The loss of virus infectivity correlated with the dryness of the sample. CMV inoculated onto paper diapers remained infectious up 48 hours; the longest infectivity survival occurred at room temperature. Pieces of a urine-containing paper diaper from a CMV-excreting infant were assayed over time for virus; CMV was determined to be infectious for up to 24 hours.

The presence of CMV in saliva and urine excretion from day care centre children was evaluated by Hutto et al (1986). Swabs taken from hand surfaces and environmental studies of objects easily accessible to the children (toys, toy boxes, and door handles) were examined to determine their role in the transmission of CMV within day care

centres. Environmental cultures were taken after a child had been observed mouthing the object. From the saliva samples, 14 out of 47 children were positive for CMV excretion, 22 out of 55 urines contained CMV, three out of 44 hands samples were positive for virus presence and two out of 70 surfaces were CMV contaminated; however, five out of seven toys mouthed by the children had CMV on the surface. CMV was determined to survive on surfaces up to 30 minutes (not tested for longer time intervals), which is more than adequate time for another child to come in contact with the contaminated surface and become infected.

A day care centre's environmental surfaces were also studied by randomly sampling for presence of CMV (Murph et al 1986). No attempt was made to determine if a child had previously contacted the surface. Although CMV excreting children were identified in the day care centre, all environmental surfaces were determined to be negative for presence of infectious virus.

3. VIRUS ISOLATION FROM DAY CARE CENTRE STAFF

Researchers evaluating the presence of CMV excretion in day care centre populations have expressed concern that a CMV-excreting child could transmit the virus to a susceptible seronegative pregnant female, thereby causing a primary CMV infection with congenitally infection effects on

the fetus. To ascertain if this was a valid concern, studies on the United States day care centre staff populations were performed to determine the prevalence of seronegative women of child-bearing potential. A seroprevalence of 53% was found for women of child bearing age in the similar age, race and social status as women working in day care centres in England (Nelson et al 1987).

Serum samples from 16 day care centre staff from the United States were studied for the presence of CMV antibodies (Pass et al 1982). Antibodies were shown to be present in 10 out of 16 day care centre staff; 50% of the women exhibiting CMV antibodies were of child-bearing age. In another study involving the staff from five day care centres, CMV seropositivity ranged from 50% to 100% (Hutto et al 1985B). The day care centres with the highest CMV excretion among the children also contained the highest percentages of seronegative staff (50% and 21%) indicating the existence of a prospective potential for virus transmission to a seronegative child-bearing day care care employee in direct contact with CMV-excreting children, with the possibility of congenitally effects to the child.

A larger survey involving 17 day care centre staff populations identified 112 out of 177 day care centre personnel to have CMV antibodies (Pass et al 1986). In the individual day care centres, the CMV antibody positive

percentage ranged from 48% to 100%, reflecting a seronegative percentage among the staff of 52% to 0%. Pass did not define the population surveyed so it is not possible to determine how many of the staff were of child-bearing age.

A survey for the presence of CMV in day care centre staff was also conducted by Adler (1988). Over an approximately two year period, twelve female day care centre employees were assessed both serologically and by virus isolation for the presence of CMV. Eight of the employees were found to be seropositive and three employees excreted the virus. Restriction endonuclease analysis confirmed that the identity of the virus isolates were the same as the predominant strain found in the day care centre children. Most likely the children infected the staff.

The number of day care centre staff CMV seroconversions was determined by an evaluation of the staff from one day care centre for antibodies to CMV, with a resurvey six months later (Murph et al 1986). Although the number of staff participating in the survey was quite small (the study consisted of five staff members) two of the five staff members were initially seropositive. Three of the five staff members were seropositive during a reevaluation six months later, indicating that one member of the staff had had a recent CMV infection. Virus cultures and restriction

endonuclease analyzed were not performed to determine if the source of the infection was a child attending the day care centre.

A comparison of CMV seroprevalence between day care centre staff and control women was undertaken by Nelson et al (1987). Day care centre staff from four day care centres were assessed by both serological analysis and by viral culture every six months for a year. The CMV prevalence for the 41 staff, ranging in age from 17 to 57 years, was compared to a control population of 500 first pregnancy women matched for age, race and social status. An overall CMV seropositivity of 66% was displayed in the day care centre staff; whereas, the control group had a 53% CMV seropositivity. No day care centre staff seroconverted; however, two CMV-excreting staff were identified. Restriction endonuclease analysis determined one of the staff's CMV isolate to be identical to the virus excreted by a pair of siblings in the day care centre where she was employed. The other staff's viral isolate was found to be unique in its restriction endonuclease pattern, possibly representing an infection source external to the day care centre.

The personnel from infant developmentally-delayed centres were compared to staff from regular day care centres to determine whether a difference existed in the CMV

prevalence (Jones et al 1985). The 130 staff members evaluated were from 16 centres, nine intellectual development centres and seven day care centres. CMV seropositivity was found in 30 out of 72 staff for the infant developmentally-delayed centres and 35 out of 58 staff in the day care centre staff. The CMV seronegative staff were resurveyed one year later to determine if any staff had been infected with CMV transmitted by a child attending the centres. Although CMV-excreting children attended the centres, no seroconversion occurred. CMV was not transmitted to any seronegative staff, possibly due to good hygienic practices by the day care staff.

4. CONCLUSIONS FROM CYTOMEGALOVIRUS STUDIES ON DAY CARE CENTRE POPULATIONS

1) The prevalence of CMV excretion within day care centre children populations has been shown to be higher as compared to both excretion and serological prevalence of CMV in home cared children; ranging from 8 to 57% in day care centre children and 7 to 17% for children cared for at home (Table 2). The CMV prevalence difference shown between day care centre children and control children cared for at home may not be an accurate representation due to the difficulties associated with obtaining proper controls (reviewed in the Discussion). Children who had attended a day care centre for at least 40 hours per week or had entered a day care

TABLE 2 Previous day care centre population CMV urine excretion surveys 1976-1988.

The surveys have been categorized into point prevalence surveys and period prevalence surveys.

AUTHOR	LOCATION	DAY CARE	CMV PREVALENCE (URINE EXCRETION)
POINT PREVALENCE CMV URINE EXCRETION SURVEYS			
STRANGERT ET AL 1976	STOCKHOLM, SWEDEN	ORIGINAL DCC	22% (13/60)
		OVERALL 13 DCCs	23% (9/40)
		HOME CARE	10% (4/40)
PASS ET AL 1982	ALABAMA, UNITED STATES	DCC	53% (36/68)
HUTTO ET AL 1985A	ALABAMA, UNITED STATES	DCC #1	41% (28/68)
		DCC #2	26% (15/58)
		DCC #3	55% (34/62)
		OVERALL 3 DCCs	41% (77/188)
		HOME CARE	15% (10/66)
HUTTO ET AL 1985	ALABAMA, UNITED STATES	DCC A	46% (27/59)
		DCC B	34% (20/58)
		DCC C	28% (13/46)
		DCC D	8% (4/48)
		DCC E	10% (2/20)
JONES ET AL 1985	SAN FRANCISCO, UNITED STATES	OVERALL 4 DCCs	22% (20/90)
PERIOD PREVALENCE CMV URINE EXCRETION SURVEYS			
PASS ET AL 1984	ALABAMA, UNITED STATES	DCC	57% (59/103)
		HOME CARE	17% (18/109)
ADLER 1985A	W. VIRGINIA, UNITED STATES	DCC	32% (21/67)
		CONTROL	7% (64/926)
ADLER 1985B	W. VIRGINIA, UNITED STATES	DCC	24% (16/66)
		CONTROL	7% (36/531)
MURPH ET AL 1986	IOWA CITY, UNITED STATES	DDC SPRING	20% (8/39)
		DCC FALL	22% (9/41)
NELSON ET AL 1987	SOUTH WEST ENGLAND	DCC A	32% (9/28)
		DCC B	46% (10/22)
		DCC C	50% (13/26)
GRILLNER & STRANGERT 1988	UNDETERMINED, SWEDEN	DCC A	38% (6/16)
		DCC B	47% (8/15)

centre at an early age were found to be more likely to acquire CMV infections (Murph and Bale 1989). The children were found to usually begin to excrete CMV during their second year in attendance. The highest CMV excretion prevalence were observed in the toddler aged group of children (Pass et al 1984, Hutto et al 1985A & B, Adler 1985A, Adler et al 1985C, Murph and Bale 1989). The increase in the excretion prevalence of CMV infection within the day care centre children was attributed to attendance in a day care centre (Pass et al 1984, Hutto et al 1985A).

2) Day care centre environmental studies determined that infectious virus was contaminating the day care centre structural surfaces such as doorhandles, children's toys, and the hands of the children, all possible routes for transmission of the virus to a susceptible child or even adult (Pass et al 1982, Hutto et al 1986). Restriction endonuclease analysis of the isolated CMV genomes obtained from the children attending day care centres have identified groups of epidemiologically linked viral isolates indicative of viral transmission within the day care centres (Hutto et al 1986).

3) In rare cases, transmission of the virus to the parents of the day care centre children and the day care centre staff had been documented by restriction endonuclease analysis of CMV isolates (Adler 1986B, Pass et al 1987).

Concern has been raised for possible transmission of the virus from a CMV-infected child attending the day care centre to a seronegative women of child-bearing potential such as the child's mother or a day care centre employee.

A number of proposals have been suggested to minimize the transmission of CMV to children attending the day care centres, the day care centre personnel and to the families of children.

a - Good hygienic practices should be followed. The Child Day Care Infectious Diseases Study Group (1985) recommended that in order to prevent CMV transmission, the day care centre staff should practice good hygiene practices; for example, handwashing after contact with any child's excretions or secretions. None of the studies published reported the hygienic guidelines in effect at the day care centre(s) being examined. Pass et al (1986) stated that there is a lack of evidence concerning the risk of CMV acquisition by day care centre staff and mothers of children attending day care centres. This conclusion was based on evidence consisting of serological analysis, the CMV seropositivity was not significantly different among mothers with children attending day care centres compared with mothers who care for their children. Therefore, CMV was not readily transmissible. Good hygiene and education of both day care centre staff and families of children attending day care centres are recommended. Bale et al (1986A & B)

further recommended that the toys mouthed by the day care children should be washed in a bleach solution.

b - Educational programs for day care centre personnel regarding CMV should be initiated. The staff should be informed about CMV, its transmission and prevention (The Child Day Care Infectious Diseases Study Group 1985.) Female employees of child-bearing potential should be informed that there may be children excreting CMV in attendance at the day care centre. Emphasis should be placed on the importance of practicing good hygiene at all times when attending infants and children (Leads from MMWR, J.A.M.A. 1985).

c - Routine serological screening of the staff for the presence of CMV antibodies is not recommended. Onorato et al (1985) further proposes that in the case of pregnant women or women planning a pregnancy, routine serological screening for CMV antibodies is not recommended due to unknown risk, unavailability of testing facilities and the unknown significance of antibodies to CMV during pregnancy. However, it is suggested that if a pregnant woman experiences a heterophile negative mononucleosis during her pregnancy, she be evaluated to determine if a primary CMV infection has occurred and counselled regarding the risks of congenital CMV in the event of a primary infection.

d - Exclusion of congenitally infected infants from centre programs is unnecessary (Bale et al 1986A & B).

D. CYTOMEGALOVIRUS GENOME AND RESTRICTION ENDONUCLEASE ANALYSIS AS AN EPIDEMIOLOGICAL TOOL

1. CYTOMEGALOVIRUS GENOME

The human CMV genome consists of a linear double-stranded DNA molecule with randomly distributed nicks and gaps (Stinski 1985). The genome has a molecular weight of 1.55×10^6 daltons corresponding to 230 kilobasepairs (kb) and could conceivably code for up to 80 proteins (Colimon et al 1985). The double-stranded CMV DNA is maintained in a duplex by hydrogen bonding and probably does not have any usual linkages such as cross-links or RNA linkers (Stinski 1985).

The genome is characterized by long and short unique regions flanked by different repeat sequences that are inverted relative to each other (reviewed in Hayward et al 1984). The majority of the genome represents unique sequences, with approximately 11% of the genome repeated twice. The Towne strain of CMV has DNA composed of a long (L) unique sequence of approximately 197 kb and a short (S) unique sequence of approximately 42 kb; combined, these two segments comprise 80 to 90% of the genome. The L and S unique sequences are flanked by smaller reiterated sequences of 11 kb and 2-2.5 kb respectively, the L segment inverted repeats are quite variable in size. The reiterated

sequences at the end of the molecule are termed terminal repeats and the internal repeat sequence referred to as internal repeats. The inverted repeats are in a reversed order for both the unique L segment and the unique S segment to constitute the molecular link allowing the DNA to form a circle. The CMV DNA is replicated from a circular intermediate form.

L and S components may occur inverted relative to each other. The viral genome occurs in four different isomers. These four subpopulations of CMV DNA molecules consist of a prototype, an inversion of S, inversion of L, and inversions of both S and L (reviewed in Stinski 1985). Somogyi et al (1986) described the HCMV four isomers of the DNA molecule. Each isomer represents one of four different arrangements and is therefore considered to be one-fourth molar (0.25 M). Restriction endonuclease cleavage at sites outside the repeat sequences would generate four fragments relative to the molarity of the intact virus DNA in 0.5 M concentrations, four fragments in 0.25 M concentrations, and all other fragments situated between the terminal and junction fragments would be present in concentrations of 1 M (Stinski 1985).

The cohesive ends of the molecule or the terminal direct repeats at the ends of the L and S unique sequences of the genome are designated the "a" sequence(s). If the L

and S terminal repeats are present internally, the same "a" sequences would be present internally. There is presumably a strong selective pressure to retain the inverted repeats and the "a" sequence(s) in both the large and small repeats (Hayward et al 1975A & B). The repeat sequences could play an important role in the orientation of the sequential viral gene expression and in the mechanism of viral DNA replication, responsible for size heterogeneity of the terminal and junction fragments. The "a" sequence of HCMV appear to have several important functions in viral replication (Somogyi et al 1986); they are necessary for L S segment inversions containing a signal for recombination and play a role in DNA replication such that concatemers formed during viral replication are cleaved to genome length fragments within the direct repeat bordering "a" sequence. The "a" sequence may be found in multiple tandem copies on either or both genomic termini and at the L S junction (Mocarski et al 1987).

The L unique/repeat boundaries within the CMV DNA for strains Davies, Towne and AD169, contain different sequences in this region and therefore probes unique for each strain may be possible. The bulk of the L unique regions equivalent to 95% of these three virus genomes contain nearly homologous sequences (Hayward et al 1984).

2. GENERAL DESCRIPTION OF RESTRICTION ENDONUCLEASE ANALYSIS AND STUDIES WITH CYTOMEGALOVIRUS

Restriction endonuclease analysis of the Herpes family viral DNA was extensively used as an epidemiological tool to determine the transmission patterns with HSV, and also as a means to identify HSV-1 and HSV-2 (reviewed by Roizman and Buchman 1979, Roizman and Tognon 1983). The methodology was adapted for use with CMV. Purified CMV DNA was incubated with a restriction endonuclease to produce distinct DNA fragment patterns following electrophoresis of the reaction mixture. Cleavage of viral DNA occurs when the restriction endonuclease recognizes a specific nucleotide sequence for that enzyme; for example, the site for *Bam*HI recognition is G*GATC C. This nucleotide sequence is in the reading form of a palindrome, that is, the deoxynucleotide bases have identical reading order when read in either direction. The enzyme cuts the DNA phosphodiester backbone at the recognition site creating fragments of the viral genome. The number of recognition sites in the viral DNA varies for each restriction endonuclease. Because the restriction endonuclease-generated fragments of the viral genome differ in their molecular weights, separation of the fragments may be achieved by gel electrophoresis to provide patterns of band fragments for each CMV isolate analyzed. Visualization of the electrophoretic pattern of DNA fragments is accomplished by either incorporating a radioisotope into the

DNA itself [tritiated thymidine, ^{32}P Phosphorus-radiolabelled orthophosphate (^{32}P) or by staining the DNA bands in the electrophoretic pattern (ethidium bromide or silver stain). An autoradiograph or photograph depending on the method of DNA visualization is then used to compare the DNA electrophoretic fragment patterns of each isolate. By analyzing the banding patterns, both in number and position of bands, a decision can be made as to whether the virus isolates are epidemiologically related or whether they were unique non-epidemiologically related strains. A major variation in the banding pattern has been defined to consist of a difference of two or more bands; representing epidemiologically distinct CMV isolates.

The CMV genome consists of approximately 230 kb. Most restriction endonucleases commonly used to analyze CMV isolates recognize six nucleotide pairs. Since approximately 0.1% of the CMV genome is represented by the restriction endonuclease cleavage sites themselves, any changes in nucleotide sequences excluded from the restriction site or additions and deletions in the genome causing a change in the molecular weight of the fragment would not be identified by this method of analysis (Peckham et al 1986). However, all epidemiological studies involving restriction endonuclease analysis of CMV genomes have shown unique patterns for non-epidemiologically related CMV strains (Huang et al 1980A & B, Adler 1985A & B, Peckham et

al 1986).

Restriction endonuclease cleavage site analysis to compare DNA purified from different isolates of CMV was first used by Kilpatrick et al (1976). The electrophoretic fragment patterns were compared to determine whether the band patterns were identical (showing that an epidemiological link exists between the viral isolates) or unique (indicating separate CMV infections). CMV isolates, 11 human and two simian or simian-related CMV isolates, were compared by restriction endonuclease analysis of the viral genomes (Kilpatrick et al 1976) using the restriction enzymes *HindIII* and *EcoRI*. All the viruses were determined to have unique electrophoretic fragment pattern. No distinction of viral fragment patterns into groups based on human isolate type could be identified.

Various CMV isolates were analyzed by restriction endonuclease digestion analysis, the DNA from various CMV isolates yielded distinct fragment patterns for each isolate (Huang et al 1980B). Considerable matching of the fragment patterns was noted among the isolates examined, but, epidemiologically unrelated isolates were found to have unique patterns. When virus isolates from individuals with recurrent CMV infections were analyzed by restriction endonuclease digestion, six out of eight isolates had identical DNA restriction patterns with the second isolate

when analyzed by *Bam*HI, *Xba*I or *Hind*III. One isolate had minor variations with *Eco*RI but not *Bam*HI. The remaining isolate was thought to represent a newly infecting strain.

Restriction endonuclease analysis of CMV genomes provides evidence to determine whether or not a CMV strain was transmitted between individuals. Prior to the introduction of this technology, serology based on a rise in the level of CMV antibodies - was used to determine virus transmission. For example, a pregnant physician with a primary CMV infection suspected that exposure to a congenitally infected infant was responsible for her CMV infection (Wilfred et al 1982). Serological evidence indicated that the pregnant physician had acquired CMV during the time she attended the congenitally infected newborn. Viral isolates from the physician, her aborted fetus and the congenitally infected infant were analysed by restriction endonuclease analysis. The restriction enzymes *Eco*RI and *Xba*I were utilized to determine whether the three viral isolates were epidemiologically related, indicating the virus was transmitted from the infected child to the pregnant physician and subsequently to the unborn fetus. The virus isolated from the congenitally infected newborn had a major difference in the electrophoretic fragment patterns, when compared to the viral isolates from the mother and fetus. However, the mother and fetus viral isolates were identical. The restriction endonuclease

analysis indicated the absence of an epidemiological link between the congenitally infected newborn and the pregnant physician.

In the epidemiological analysis of HSV isolates, the use of three restriction endonuclease enzymes has been determined by Roizman and Buchman (1979) to be sufficient for identification of whether an epidemiological link exists between isolated viruses. Most CMV researchers agree that the use of three restriction endonucleases will establish presence of epidemiologically related isolates in the epidemiological analysis of CMV isolates (Table 3).

Huang et al (1980B) used four enzymes, *EcoRI*, *HindIII*, *BamHI* and *XbaI* to study CMV isolates from mothers and their children. If the DNA from viral isolates showed different electrophoretic fragment patterns following digestion by these enzymes, the viral isolates were considered to be different; that is, epidemiologically unrelated. All enzymes, except *EcoRI*, were able to reveal epidemiologically related or different strains. In the case of the enzyme *EcoRI*, minor variations (the loss, addition, or movement of a band) were seen in the patterns of epidemiologically linked isolates. Among the strains that were determined to have different electrophoretic fragment patterns, major differences were found in the number of fragments and in the migration patterns between all the viruses. Since Huang et

TABLE 3 Number and identity of restriction endonucleases utilized in reported analyses of CMV isolates.

Most researchers employ three restriction enzymes during the analysis of CMV isolates. The enzymes most commonly used are BamHI, EcoRI and HindIII.

AUTHOR AND YEAR	RESTRICTION ENZYMES
HUANG ET AL 1980 A & B	BAMHI, ECORI, HINDIII AND OCCASIONALLY XBAI
VILPERT ET AL 1982	ECORI, XBAI
YOW ET AL 1982	BAMHI, HINDIII
HUTTO & PASS 1984	BAMHI, HINDIII
SPECTOR ET AL 1984	HINDIII, BGLII, ECORI
ADLER 1985A	BAMHI, BGLII, HIND III, (HYBRIDIZATION ECORI)
ADLER 1985B	HYBRIDIZATION ECORI
EMBIL ET AL 1985B	BAMHI, ECORI, XHOI, HINDIII, KPMI, PSTI, SSTI
GARRETT & WARREN 1985	ECORI, HINDIII, BGLII
HANDSFIELD ET AL 1985	BAMHI, HINDIII, ECORI
WERTHEIM ET AL 1985	ECORI, HINDIII
ADLER 1986A	ECORI, BAMHI, HINDIII, XBAI
CHANDLER & MCDUGALL 1986	HINDIII, ECORI
GRILLNER & STRANGERT 1986	BAMHI, ECORI, HINDIII
GRUNDY ET AL 1986	BAMHI, ECORI, HINDIII
MC FARLANE & KOMENT 1986	BAMHI, ECORI, HINDIII, PSTI, XHOI
GRILLNER ET AL 1988	BAMHI, ECORI, HINDIII

al's (1980A & B) original use of restriction endonuclease analysis, numerous researchers have used restriction endonuclease digestion in various studies to evaluate the epidemiological relatedness between various CMV isolates (Table 4).

As stated previously minor variations in the electrophoretic fragment patterns were found when the enzyme *EcoRI* was used in the epidemiological analysis of CMV isolates from mother and baby pairs or repeat isolates from an individual (Huang et al 1980A). A mother with a previous CMV congenitally infected baby gave birth four years later to a second child. DNA from virus isolated from the congenitally infected child, the mother three years after that child's birth, the second child at its birth and the mother two years after the second birth had identical electrophoretic fragment patterns after cleavage with the enzymes *BamHI* and *HindIII*. However, minor variations between the two maternal isolates and the two children's isolates were found when the enzyme *EcoRI* was used. This finding was repeated with another case involving isolates from a mother and child. The isolates were cultured from the mother two years prior to the child's birth, the child at birth and the mother three years after the child's birth. The DNA from the second maternal isolate had minor variations in the electrophoretic fragment patterns with the enzyme *EcoRI*. Wilfert et al (1982) also found minor

TABLE 4 Reported restriction endonuclease analyses to establish epidemiological relationships among the CMV isolates.

This table represents a summary of current literature involving the use of restriction endonuclease analysis to identify epidemiological relationships among CMV isolates.

RESEACHERS	ORIGIN OF CMV ISOLATES	EPIDEMIOLOGICAL RELATIONSHIP
WILFERT ET AL 1982	CONGENITALLY INFECTED INFANT PHYSICIAN	NONE
SPECTOR AND SPECTOR 1984	INFANT MONOZYGOUS TWINS	NONE
SPECTOR ET AL 1984	HOMOSEXUAL MEN	IDENTICAL
GRILLNER AND BLOMBERG 1984	EPIDEMIOLOGICAL-UNRELATED PATIENTS	NONE
	CONGENITALLY INFECTED CHILD MOTHER	IDENTICAL
ENBIL ET AL 1985B	CHILD'S MIDDLE EAR & URINE	IDENTICAL
GARRETT AND WARREN 1985	MOTHER'S MILK & INFANT ALSO 3 SIBLINGS	IDENTICAL
PECKHAM ET AL 1986	CONGENITALLY INFECTED BABY 13 OTHER CONGENITALLY INFECTED INFANTS CARED FOR BY BABY'S MOTHER	NONE
	7 CHILDREN ATTENDING A NURSERY	2 IDENTICAL SIBLINGS
	9 MOTHER AND INFANT PAIRS	8 IDENTICAL 1 NONE
GRUNDY ET AL 1986	SEROPOSITIVE KIDNEY RECIPIENTS	IDENTICAL
McFARLANE & KOHENT 1986	MAN'S URINE AND SEMEN	NONE
GRILLNER ET AL 1988	CONGENITALLY INFECTED CHILDREN	NONE

variations in electrophoretic fragment patterns of DNA from epidemiologically linked CMV isolates when the restriction endonuclease *EcoRI* was utilized.

Several researchers have raised the issue of whether CMV isolates from patients with similar symptoms, for example congenitally infected infants with neurological sequelae, have a common marker in the restriction endonuclease electrophoretic patterns of the digested viral DNA (Kilpatrick et al 1976, Grillner et al 1988). The restriction endonuclease patterns of CMV isolated from eight congenitally infected infants with neurological sequelae were analyzed by the enzymes *BamHI*, *EcoRI*, and *HindIII* to determine whether a common recognizable band could be identified. No evidence for such a marker was found, however, the sample size was too small for definitive conclusions and also lacked children with acquired CMV infections for comparison (Kilpatrick et al 1976).

3. CYTOMEGALOVIRUS PROBE HYBRIDIZATION

Researchers studying the epidemiology of CMV by the restriction endonuclease digestion of the viral genome have been frustrated by the length of time involved in purifying the viral DNA sample - often over six months from the original clinical specimen isolation. Spector et al (1985) used junctional hybridization as a means of determining the

epidemiological relatedness of viral isolates in considerably less time. The *EcoRI* fragments F or H from the L-S junction fragment (where the unique L and unique S fragments meet) have been shown to have complete heterogeneity for all unique CMV strains. An *EcoRI* restriction endonuclease digestion of the genomic DNA from the clinical samples is performed. The cleaved sample DNA is allowed to hybridize to the L-S junction fragment. If hybridization occurs the isolates are epidemiologically related. The procedure requires considerably less DNA, therefore, less time for viral propagation is required. However, this analysis to determine whether epidemiological links exists between viral isolates only utilizes one restriction endonuclease, whereas researchers studying the epidemiology of CMV recommend that at least three restriction endonucleases be used to determine identity between CMV isolates.

A method of DNA *in situ* hybridization using ³²P-labelled plasmids containing cloned fragments of Towne DNA was devised by Adler (1985A). A considerably smaller amount of CMV DNA was required, so less time elapsed between virus isolation and propagation of sufficient DNA for analysis. This probe technique was reported to enhance the sensitivity by identifying different nucleotide sequences present in the electrophoretic fragment patterns with identical intensities.

Although time efficient CMV probe methodologies have been developed, researchers studying CMV transmission in day care centre populations usually employ the restriction endonuclease analysis technique using either ^{32}P -radiolabelled orthophosphate incorporation or ethidium bromide DNA staining of the DNA fragments to visualize the electrophoretic fragment patterns. In Adler's recent studies (1985A and 1988), the probe hybridization technique to identify epidemiological relationships among the CMV isolates was not used, but rather ^{32}P -radiolabelling of the CMV DNA has been employed.

E. STUDY RATIONALE

CMV infection has a worldwide distribution. The age at which an individual first becomes infected with CMV depends on a number of factors, such as the geographic location, the socioeconomic status of the individual and attendance in a day care centre, all seeming to reflect the crowding factor of a population. Recently, a number of studies examining day care centre populations in the United States, England and Sweden have described the presence and transmission of CMV in day care centre children (discussed previously). Concern has been expressed regarding the possible consequences following the exposure of children and seronegative pregnant females to children excreting the virus. Recommendations regarding CMV in the day care centre environment have been proposed (See page 78). The prevalence of CMV in the general Halifax population is among the lower CMV prevalences reported for developed nations (Embil et al 1969A). Data is required to determine whether high CMV excretion prevalence found in United States day care centre children is also exhibited by Halifax day care centre populations.

The approach to this study to determine CMV shedding and transmission among children attending Halifax day care centres involved a cross-sectional survey of Halifax day care centre populations to estimate the prevalence of CMV

urine excretion. A one-time sampling of the children was used to collect the urine specimens to isolate the virus. Results of the virus isolation were analyzed with the demographic information collected from the parents of the children to determine whether risk factors existed for CMV excretion by the day care centre children. The CMV isolates were analyzed by restriction endonuclease analysis to identify whether epidemiological relationships existed among the virus isolates. CMV transmission data was obtained by repeatedly sampling the population attending one of the day care centres during three surveys.

In order to perform the study cooperation from the Department of Social Services, Day Care Division, day care centre staff and the children and parents of child attending centres was essential. The Department of Social Services, Day Care Centre Division was contacted to obtain authorization for the survey to be conducted. Meetings with the staff from the each of the day care centres selected to be surveyed were held to obtain the staff's cooperation and to explain the details of the study. Parents of the children attending day care centres were solicited to allow their children to participate in the survey, as well as to provide information on the family's demographic descriptors. The presence of CMV within day care centre populations was assessed by urine analysis rather than by presence of CMV antibodies, due to the invasive procedure required to

collect blood and the need for the virus to be isolated for further analysis.

A number of issues were to be addressed during this study. The major issue concerned the prevalence of CMV in Halifax day care centre children populations and the possibility of CMV transmission within day care centre populations. The information obtained was then to be used to examine questions such as whether Halifax day care centre populations differ in CMV prevalence from those found in the United States, Britain and Sweden; whether any of the possible factors by which the Canadian day care centres differ from the other countries were responsible for any observed deviations in CMV prevalence; whether risk categories for infection by CMV could be identified for the day care centre populations; and whether recognizable strains of CMV prevalent within the day care centre population could be identified.

The issue of whether Halifax day care centre children have a higher CMV prevalence than the general Halifax children population was not directly assessed. The CMV prevalence in day care centre children was, however, compared to a previous study of the CMV prevalence in the general Halifax children population [as determined by antibody levels for Halifax children in 1968 by Embil et al (1969A)]. Although the CMV prevalence in this study was

determined by urine analysis, Pass et al (1982) in their comparison of CMV prevalences obtained by both analyses used a general estimate of CMV antibody levels made by adjusting the CMV urine excretion prevalence by 10% to 30%.

The issue of whether the recommendations previously published for United States day care centres are appropriate for Halifax day care centres was to be examined. Recommendations for prevention of CMV infections within day care centre populations are based on United States findings. The CMV prevalence for Halifax day care centre populations has not been determined; therefore, the applicability of any proposed recommendation to Halifax day care centre populations is unknown.

This study has several advantages over the previously reported studies. CMV excretion prevalences for Halifax children aged up to six years attending day care centres were determined during the study. Previous studies on Halifax children populations of similar ages were based on serological studies (Embil et al 1969A). Therefore, the numbers of children who would be able to transmit the virus - that is, excreting virus - were not assessed.

The design of this study is longitudinal. This allowed information regarding CMV excretion by day care centre children over extended periods of time to be obtained; as

well as, determining whether transmission of CMV occurred among the day care centre children.

CMV transmission would be able to be directly assessed by restriction endonuclease digestion analysis of the virus genome rather than indirectly associating virus transmission with high CMV prevalence. The progression of virus transmission could be identified. At least three restriction enzymes would be used for the epidemiological analysis.

The study yielded information regarding CMV prevalence and transmission in day care centre children directly applicable in assessing the validity of the proposed recommendations regarding CMV in the day care centre environment for Halifax day care centres.

The remainder of this thesis is comprised in five sections: the Materials and Methods in which the procedures used to collect then analyze the data and the samples from the day care centre children; the Results which encompass the description of the day care centres and their populations, the virus excretion prevalence, the risk factor analysis and the molecular epidemiology of the isolates; the Discussion in which the results of the study on CMV excretion and transmission in the surveyed Halifax day care centre populations is discussed, as well as, comparing the

obtained results to the reported literature, determining whether risk categories for CMV excretion could be found, and describing the molecular epidemiology of CMV in day care centre A; and the Conclusions of this study.

II. MATERIALS AND METHODS

The Materials and Methods of the CMV excretion prevalence and transmission study within Halifax, Nova Scotia day care centre children are described in five sections; A - population studied, B - cell culture techniques, C - virus culture techniques, D - restriction endonuclease analysis techniques, and E - statistical analysis.

A. POPULATION STUDIED

The aim of this project was to determine the prevalence and transmission of CMV among the children attending several Halifax day care centres. Because the project involved human subjects, ethical approval was sought from and granted by both the Research Committee of the Izaak Walton Killam Hospital for Children and the Faculty of Medicine Ethics Review Committee of Dalhousie University. Project approval by the Province of Nova Scotia Department of Social Services, Day Care Division and the Province of Nova Scotia Department of Health was also obtained following various meetings with representatives of these departments. The Department of Social Services, Day Care Division provided information regarding the day care centres within the

Halifax-Dartmouth area including the location of the day care centres, the name of the day care centre director, the number of children in attendance, age range of the children, and whether the day care centre contained children from families receiving social assistance to subsidize the cost of the day care centre services. According to the Nova Scotia Department of Social Services, Day Care Division, a day care centre must meet licensing requirements which insist on 30 square feet per child, one toilet and sink per 10 children and no more than 25 children per day care centre attendant (Personal communication Nova Scotia Department of Social Services, Day Care Division).

In the Halifax-Dartmouth area during the year 1984, there were 92 day care centres listed with the Nova Scotia Department of Social Services, Day Care Division. These day care centres were classified into 23 registered day care centres and 69 private day care centres. The only difference between the two types of day care centres is that a registered day care centre will accept children whose families receive a subsidy from the Nova Scotia Department of Social Services, whereas, a private day care centre will not accept children whose parents are subsidized. However, all families using the services of a registered day care centre do not necessarily qualify for the social assistance and the amount of the subsidy is based on the individual family's financial need. The subsidy is defined as

financial assistance provided to families which meet specific criteria defined by the Income Test and the Community Board determined by the Nova Scotia Department of Social Services. The Nova Scotia Department of Social Services maintains strict confidentiality regarding the identity of the families receiving subsidies and the amount.

A geographic subdivision classified the day care centres in the Halifax-Dartmouth area according to location. In Halifax there were 19 registered day care centres and 41 private day care centres. The centres located in the Dartmouth area consisted of four registered day care centres and 21 private day care centres. A further subdivision can be made using Statistics Canada census tracts. The Halifax-Dartmouth area is classified into 45 census tracts (Statistics Canada 1982). The majority of the Halifax day care centres are located in census tracts 004 and 006. These census tracts, however, do not contain the largest numbers of families (Figure 9).

The number of children that each day care centre accepts varies between six to 95 children (Figure 10). Although the majority of the centres accept from 10 to 19 children, the mean number of children per day care centre was calculated to be 30 children per day care centre. The number of children enrolled per private day care centres was found to be lower than in the registered day care centres.

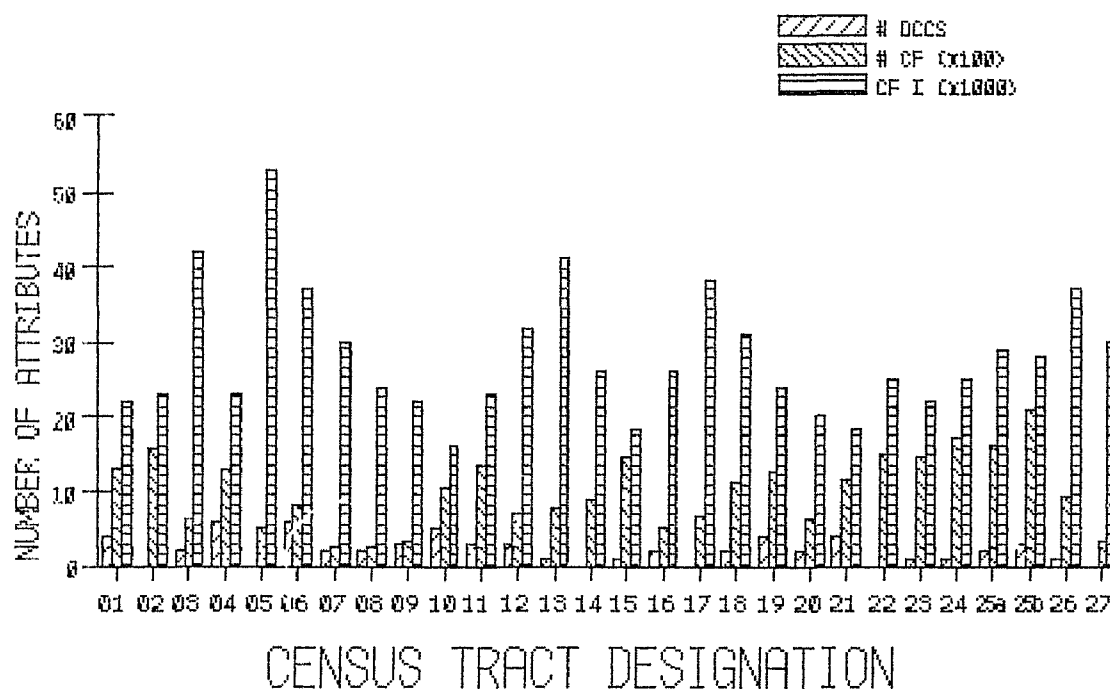


FIGURE 9 Halifax census tracts by number of day care centres (# DCC), number of census families (# CF x100) and family income (CF I x1000). Data adapted graphically from the 1981 Census (Statistics Canada 1982). Census tract 25 has been divided into two separate census tracts by Statistics Canada, 25a represents tract 25.01 and 25b represents tract 25.02.

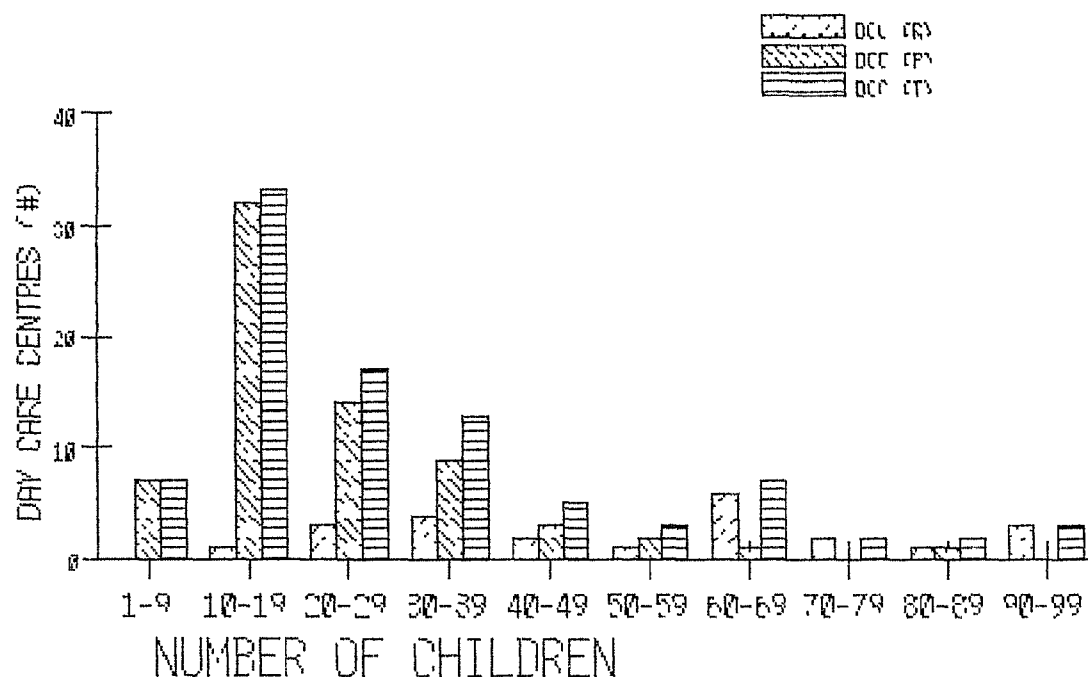


FIGURE 10 Registered and private day care centres in the Halifax-Dartmouth area by number of children in attendance was compared. Data supplied by the Nova Scotia Department of Social Services Day Care Division (adapted graphically).

- (P) - Private day care centres
- (R) - Registered day care centres
- (T) - All day care centres

The majority of registered day care centres may accept between 60 and 69 children: some accept as many as 90 to 99 children.

After the meetings with the Nova Scotia Department of Social Services, Day Care Division the inability to classify the day care centres according to the basis of socioeconomic factors became apparent. Social assistance was not given to the day care centre itself but rather to the individual families. Therefore, while the day care centres which accepted children whose families received assistance could be determined, the Department would not identify the number of families within the day care centre receiving assistance. The location of the day care centre might have provided a basis for socioeconomic identification; however, through discussions with the directors and other people involved, it was determined that many families bring their children to a day care centre near their place of employment (as shown previously by census family data versus location of day care centres, Figure 9). Thus, the day care centre population may not reflect the socioeconomic status of the geographic area in which the day care centre was located. Therefore, because there was a representative sample of both classifications of day care centres and day care centre sizes located around the university, the selection of the day care centres included in this project was based on proximity to Dalhousie University. The day care centres

chosen varied in number of children from quite large (76 children) to quite small (17 children) so a representative centre from each of the size ranges would be included in the survey.

Once the day care centres to be surveyed were selected, meetings with the individual day care centre directors were held in order to seek their approval. Discussions were held with the day care centre staff on the collection of the urine from the children. The method used was dependent on the age and toilet-training of the individual child. Details regarding the storage of the urine until transfer to the laboratory were also discussed. Opinions and suggestions on various aspects of the collection procedures were solicited from the day care centre staff, and the progress using these procedures was constantly reviewed. One requirement for ethical approval of this study was that the day care centre staff collected the specimens. The staff was familiar with the children and their urinary habits through routine daily contact. This lessened the likelihood of a child being subjected to undue trauma which may have occurred if a stranger collected the urine. If a child refused to provide a urine sample after three separate requests, the child was omitted from the survey. Thus, the child would not be subjected to stress produced by repeated requests for a specimen.

Three of the day care centres selected to be included in the survey were operated by Dalhousie University, the remaining three centres were privately operated. All six centres were licensed by the Nova Scotia Department of Social Services, Day Care Center Division. To preserve the day care centre's anonymity, the day care centres were coded A to F. Registered day care centres were coded A to C and private day care centres were coded D to F. Survey and consent forms along with a letter describing the study were sent to each child's parents (Appendix II). The letter informing the parents was drafted with the help of members from the Dalhousie University Departments of Microbiology (Dr. J. Embil), Community Health and Epidemiology (Dr. F. White), and Health Services (Dr. J. Johnson), as well as the Nova Scotia Department of Social Services, Day Care Center Division (Mrs. Beissett-Sagar) and one of the day care centre directors.

The size of the six day care centres included in the survey ranged from 17 to 76 children. The study population consisted of children aged from nine months to six years attending these day care centres. Information collected by the survey form included: the child's home address, age and sex, the parent's age, education and occupation and information on siblings if any, their age and sex. The consent form had to be signed by one of the children's parents in order for that child to be included in the study.

A phone number was included in the letter sent to the parents for inquiry purposes. The followup to the nonresponding parents consisted of repeat letters and forms sent approximately one month later in hopes of increasing the response. This followup procedure was not carried out at day care center E due to the refusal of the director. The director felt the parents had indicated a negative response by not responding to the survey forms. Urine specimens were collected three to four weeks after the initial survey forms had been distributed. The delay allowed time for the parents to return the consent forms.

Day care centre A consisted of four divisions of children designated "infant" (aged 12 to 18 months), "toddler" (aged 18 to 30 months), "junior" (aged 31 to 42 months) and "senior" (aged 43 to 60 months). Each age group had a separate room; however, some rooms were linked by corridors. The infant group was isolated from the other children and all facilities required for their maintenance, including washing and diaper-changing areas, were located in their area. The toddler and junior group of children shared washroom facilities; whereas, the senior children had a separate washroom facility. An outdoor playground was shared by the children. Considerable mixing of the children occurred during the day, particularly amongst the toddler and juniors groups.

Day care centre B also was composed of discrete rooms for each age group, unit I (under 19 months), unit II (19 to 42 months) and unit III (43 to 60 months). These rooms were not interconnected; however, there was a common playground in which the children could congregate.

Day care centre C embodied separate care areas for each age group of children. No infants attended the day care centre. The junior and senior groups were attended in distinct areas of a common room and all the children shared washroom facilities. No playground was available to the children.

Day care centre D was composed of two separate rooms for two groups of children. The children were grouped without regard for age. Each room had its own washroom facilities. Although this day care centre did not have an outdoor playground, considerable common areas were available to the children i.e. a gymnasium and pool.

Day care centre E consisted of one common area to care for the children - the basement of a previous family dwelling. No playground was available to the children.

Day care centre F had one common area for all age groups of children.

Day care centre F was omitted from the CMV prevalence survey due to the hostile parental response to the survey forms. Considerable pressure was placed on the day care centre director to have the study halted. However, the data collected for the day care centre descriptors was used in the statistical analysis to determine whether differences could be found to exist between registered day care centres and private day care centres.

Data from the five remaining day care centres were used to estimate the excretion prevalence of CMV and also to determine whether risk factors could be identified for CMV excretion within the day care centre populations. In addition, the largest day care centre was also used to determine whether transmission of CMV occurred amongst the children. The transmission data were collected by repeated surveys between December 1984 and April 1987, during which time three samplings were conducted. The centre was selected due to the staff cooperation and the number of children in attendance. The CMV urine excretion prevalence for the children attending day care centres was estimated by determining the number of children excreting the virus then dividing that number by the total number of children from which urine samples were obtained.

B. CELL CULTURE TECHNIQUES

The human neonatal foreskin fibroblast (HNFF) cell monolayer culture system devised by Embil and Faulkner (1964) was used in this CMV prevalence and transmission survey for virus isolation and cultivation.

1. HUMAN NEONATAL FORESKIN FIBROBLAST EXPLANT CELL CULTURE

The human neonatal foreskin tissue was obtained from routine circumcisions performed at the Grace Maternity Hospital in Halifax, Nova Scotia. Surgically excised tissue was placed in Eagle's Diploid Medium (EDM) (Gibco Canada, Grand Island Biological Company of Canada, Appendix III) with 1% Garamycin Reagent Solution (GRS) (Garamycin Reagent Solution: gentamicin sulfate, USP Schering Corporation) and transported to the laboratory. The tissue was positioned in a sterile Petri dish and minced with two sterile scalpels. The specimen was washed in 10 ml EDM with 1% GRS and centrifuged (International Centrifuge Refrigerated Model PR-J, International Equipment Co.) at low speed (1000 rpm) for 5 minutes at 4°C. The supernatant fluid was decanted then replaced with 10 ml EDM supplemented with 10% Fetal Calf Serum:Calf Serum (1:1) (FCS:CS)(Fetal Bovine Serum, Flow Laboratories; Calf Serum, M.A. Bioproducts) and 1% GRS and centrifuged for 5 minutes at low speed (1000 rpm). After the pellet was transferred to a 75 cm² sterile plastic

Falcon flask (Falcon Division, Becton Dickinson and Company), a sterile Pasteur pipette was used to evenly distribute the minced tissue. The plugged flask was then incubated overnight at 36°C in an atmosphere of 5% CO₂. The following day 40 ml of Growth Medium (GM), that is EDM supplemented with 10% FCS:CS, was introduced into the flask and the tissue was incubated until a complete monolayer had formed. During this incubation the medium was changed weekly.

When the cell monolayer was confluent the medium was discarded. The cell monolayer was washed with 5 ml Phosphate Buffered Saline (PBS) (Appendix III) which was then removed. A volume of 5 ml sterile 0.25% trypsin (Appendix III) was added, and the flask was incubated at 36°C in an atmosphere of 5% CO₂ until the cells had dispersed - approximately 10 to 15 minutes. The cells were transferred to a sterile 175 cm² containers (glass Blake bottles and 175 cm² sterile Falcon flasks (Falcon Division, Becton Dickinson and Company were used interchangeably) and 70 ml GM was added. This flask was labelled passage #1 (P-1). Once the cell monolayer was confluent, the monolayer was trypsinized (as above) and transferred to two sterile Blake bottles with GM.

Once a cell line had been established, the culture was maintained as a monolayer with weekly medium changes of

Maintenance Medium (MM), that is EDM supplemented with 5% FCS:CS. These stock cultures were used to make additional monolayers of HNFF cells contained in Blake bottles or to make culture tubes of HNFF cell monolayers. Portions of every established cell line were stored in liquid nitrogen (as described below) in order to maintain a readily available supply of cells for cell culture.

2. HUMAN NEONATAL FORESKIN FIBROBLAST CELL CULTURE

a. AMPLIFICATION OF BLAKE BOTTLES CONTAINING CELL MONOLAYERS

When the need arose to increase the number of HNFF cell monolayers, the following procedure was utilized. The medium from a Blake bottle containing a confluent HNFF cell monolayer was removed. The monolayer was washed with 10 ml of PBS which was then removed and replaced with 8 ml of sterile 0.25% trypsin. The flask was incubated at 36°C in an atmosphere 5% CO₂ until the cells dispersed. The cells/trypsin solution was transferred to two Blakes bottles and 70 ml GM was then added to each. When the number of HNFF cell monolayers was being increased, usually each HNFF cell monolayer contained in a Blake bottle was subdivided to two new Blake bottles. However, if the demand for new HNFF cell monolayers exceeded the number of Blake bottles containing cell monolayers available via this procedure then

the original Blake bottle was subdivided 1:4.

b. PRODUCTION OF CELL CULTURE TUBES

Tubes of HNFF cell monolayers were also made by the trypsinization of a confluent HNFF cell monolayer contained in a glass Blake bottle by the previously described method for amplification of cell monolayers. However, after the trypsinization of the cell monolayer, the cells were placed in a sterile flask containing 60 ml of MM. The cells were distributed through the medium, as evenly as possible, using a magnetic stirring bar. Approximately 1.2 ml aliquots of the cell suspension were then transferred into 50 sterile cell culture tubes (125 mm x 17 mm, Kimax, Kimble Products). The tubes containing the cells were incubated at 36°C in an atmosphere of 5% CO₂ until confluent monolayers had grown, (approximately four to five days). Once the monolayers were confluent, the cell culture medium was discarded and replaced with Cell Culture Tube Maintenance Medium, EDM supplemented with 2% FCS:CS then incubated at 36°C in an atmosphere of 5% CO₂. The medium was replaced with fresh medium in one week intervals.

3. LIQUID NITROGEN STORAGE OF HUMAN NEONATAL FORESKIN FIBROBLAST CELLS

a. FREEZING HUMAN NEONATAL FORESKIN FIBROBLAST CELLS

All established cell lines were stored for future use and reference in liquid nitrogen by the following procedure. A Blake bottle containing a confluent cell monolayer was trypsinized by the previously described method. The trypsinized cells were suspended in 10 ml of EDM and centrifuged at 1500 rpm (low speed) for 10 minutes. The resultant pellet was suspended in 3 ml Cell Freezing Medium, EDM supplemented with 40% FCS:CS and 8% Dimethylsulfoxide. One ml of the suspension was placed into each of three 2.0 ml vials (Pro-Vial, Dynatech Laboratories Inc.). The cell line, passage number and date frozen were inscribed on the vials and also recorded in a permanent records book. The vials were then placed in an aluminum cane (Edwards Ari-Supply Inc.) and lowered into a 5°C alcohol bath. The temperature of the bath was reduced 1°C per minute until the temperature reached -25°C by the addition of small pieces of dry ice. At this point the temperature was reduced 5°C per minute until the final temperature of -70°C was reached. The cane was then transferred to the liquid nitrogen for storage. An alternate method was also employed. The prepared vials of cell suspension were packed in styrofoam chips within a styrofoam box. The box was then placed in a

-20°C freezer overnight. The following day the vials were transferred to the aluminum cane holders and lowered into liquid nitrogen. No major differences in cell viability were seen between the two methods.

b. REACTIVATING FROZEN HUMAN NEONATAL FORESKIN FIBROBLAST CELLS

When the need arose to use a cell line stored in the liquid nitrogen, a vial containing the frozen cells was removed and placed in a 37°C water bath for approximately one minute. The cells were then transferred to 10 ml EDM and centrifuged for 10 minutes at low speed (1500 rpm). The resultant pellet was suspended in a small volume then transferred to a plastic 75 cm² Falcon flask containing 40 ml GM. The flask was incubated at 36°C in an atmosphere of 5% CO₂ until a confluent monolayer was obtained.

C. VIRUS CULTURE TECHNIQUES

1. CYTOMEGALOVIRUS ISOLATION

The urine samples obtained from the children attending day care centres in the Halifax area of Nova Scotia (as described previously) were cultured for the presence of CMV. Urine specimens were collected directly in sterile Pediatric Urine Containers (Seamless Hospital Products Company) or indirectly by first collecting the urine in sterile urine collectors then transferring the specimen into a sterile urine specimen container. The urine collection was performed by the day care center staff and the specimens were kept in an ice packed container until processing. A 0.5 ml urine sample was diluted in 1 ml of EDM with 1% GRS, and subsequently 0.5 ml of the processed sample was inoculated onto each of two HNEF cell monolayers contained in cell culture tubes. The cultures were incubated at 36°C in an atmosphere of 5% CO₂ for up to two months. Maintenance of specimens was achieved by weekly changes of the medium and a microscopic examination for signs of CMV CPE.

2. DETECTION OF CYTOMEGALOVIRUS

Presence of virus was detected by examining the cell culture tubes inoculated with the various specimens under

40X magnification for evidence of typical CMV CPE. If characteristic CPE (Embil et al 1969B) was recognized, the cell culture specimen tubes were segregated from the remaining negative tubes and maintained separately to prevent CMV or bacterial cross-contamination during routine medium changes.

3. CYTOMEGALOVIRUS PROPAGATION

Once the virus inoculated HNFF cell monolayer manifested the characteristic CPE for CMV, maintenance was performed by weekly changes of medium until approximately 50% of the cell sheet showed CPE. This was considered sufficient to propagate the virus by transferring the entire specimen tube monolayer to a plastic 75 cm² Falcon flask containing a HNFF cell monolayer and GM. The cell culture tube monolayers were washed with 2 ml of PBS. Approximately 0.5 ml of 0.25% trypsin was added onto the monolayer and left at room temperature until the CMV cell monolayer had dispersed. The dispersed cells were then transferred to a plastic Falcon flask containing a HNFF cell monolayer, then incubated at 36°C in an atmosphere of 5% CO₂ until the cell monolayer exhibited 50 to 70% viral CPE. At this point the CMV infected cell monolayer was trypsinized and divided among four HNFF cell monolayers contained in Blake bottles. Part of the virus recovered from these Blake bottles was used for viral DNA isolation for restriction endonuclease

analysis and the remainder was frozen and stored in liquid nitrogen for reference.

4. LIQUID NITROGEN STORAGE OF CYTOMEGALOVIRUS

The procedure for liquid nitrogen storage of CMV, both freezing and reactivation of the HNFF cells infected with CMV, was identical to the procedures used for the storage of HNFF cell monolayers. The virus culture was frozen when approximately 70 to 80% of the HNFF cell monolayer exhibited CMV CPE. After thawing, approximately 0.1 ml of the virus-cell suspension was also inoculated to a plastic 75 cm² Falcon flask with a confluent HNFF cell monolayer.

D. RESTRICTION ENDONUCLEASE ANALYSIS TECHNIQUES

The CMV isolates were analyzed for epidemiological relationships by restriction endonuclease analysis techniques. Two restriction endonuclease analyses were utilized during the study: the tritiated thymidine DNA labelling technique and the ethidium bromide DNA staining technique.

1. TRITIATED THYMIDINE TECHNIQUE FOR LABELLING

CYTOMEGALOVIRUS DNA AND DNA EXTRACTION

CMV from which the DNA was to be isolated was grown in HNFF cell monolayers in a 175 cm² plastic Falcon Flask. Once approximately 60% of the cell monolayer exhibited CPE, the medium was removed and replaced with 20 ml EDM with 2% FCS:CS. A volume of 400 μ l [4 microCurie (uCi) per ml of the culture medium] of tritiated thymidine (thymidine [methyl-1,2-³H]-90-110 Ci/mmol, New England Nuclear) was added to the virus culture. The infected monolayers were further incubated until 80 to 90% CPE was seen. The supernatant was discarded and 2 ml of HIRT Buffer (Appendix III) was placed on the infected monolayer. The flask was rocked for 15 to 45 minutes at room temperature until all of the cells were lysed and the solution was viscous. One-quarter volume of 5 M NaCl was slowly added to the supernatant. After overnight refrigeration at 4°C, the

solution was centrifuged at 15,000 rpm (30,000 x g) at 4°C in a B-20 centrifuge (I.E.C.) for 15 minutes. The supernatant was collected, 5 ml EDTA-NaCl saturated phenol (Appendix III) was added, mixed, and the solution centrifuged at 15,000 rpm (30,000 x g) at 4°C in a B-20 centrifuge (I.E.C.) for 15 to 30 minutes. The aqueous layer was extracted twice with approximately 2 volumes of anhydrous ether (Fisher Scientific Ltd). After the second extraction the sample was allowed to stand in a fume hood for 1 to 2 hours at room temperature, then dialyzed overnight at 4°C against Dialysis Buffer (Appendix III). The dialyzed sample was aliquoted in Eppendorf tubes and stored at -20°C until required.

The radioactivity of the sample was determined by placing a 5 ul sample on a 2.4 cm diameter glass filter paper (Whatman Glass microfibre filters, Whatman Ltd). The filter was washed three times with 50 ml 10% weight/volume trichloroacetic acid to precipitate the DNA and dried under an infrared light. The filter was then placed in 10 ml of scintillation fluid [2,5-bis-2 (5-tert-butylbenzoxazolyl) triophene Scintillation Grade Packard Instrument Company Inc.] and the radioactivity counted for one minute in a scintillation counter (Isocap/300 Liquid Scintillation System Nuclear). Spectrophotometric analysis for DNA content of the sample was not performed on these tritiated thymidine labelled DNA samples. The intensity of the

electrophoretic fragments produced by restriction endonuclease analysis of the samples was dependent on the radioactivity of the sample, not the DNA concentration.

2. DIGESTION AND ELECTROPHORESIS OF DNA

The necessary volume of viral DNA to be analyzed depended on the radioactivity in the sample. A volume of sample ranging from 5 ul to 87 ul was placed in a Eppendorf tube with 10 ul of the appropriate 10 times (X) concentrated enzyme buffer (Appendix III), 3 ul of restriction enzyme (Bethesda Research Laboratories) [BamHI (10 units (U)/ul), HindIII (10 U/ul) KpnI (5 U/ul), SalI (10 U/ul) and XhoI (10 U/ul)] and sterile distilled water to make the volume to 100 ul. The samples were incubated at 37°C for approximately 3 hours after which 5 ul of stop buffer (Appendix III) was added to the sample, mixed gently, then placed into a 100 ul well of a 1% agarose gel in TEA buffer. The voltage (EC 452 Power Pack, E-C Apparatus Corporation) was adjusted to 55 V for the first 1/2 hour then lowered to 35 V overnight (16 to 16 1/2 hours). The buffer used during the electrophoresis was TEA (Appendix III).

Following electrophoresis, the gel was placed in EN³HANCE (New England Nuclear) and rocked for approximately 3 hours after which it was rinsed and rocked 1 hour in water. The gel was dried on a Bio-Rad (Bio-Rad

Laboratories) gel dryer for 1 hour with heat applied during the final seven minutes. The dried gel was placed in a Kodak X-Ray Exposure Holder (Eastman Kodak Company) in contact with a Kodak X-Omat AR Film 14 (Eastman Kodak Company) and exposed for 4 to 6 weeks at -70°C .

The film was developed for five minutes (Pik Developer and Replenisher, Picker Chemical Inc.), after which it was briefly rinsed in water and placed in fixer (Kodak Rapid Fixer with Hardner, Eastman Kodak Company) for five minutes. The developed film was rinsed with Photoflow solution (Eastman Kodak Co) and air-dried.

3. ETHIDIUM BROMIDE STAINING TECHNIQUE FOR RESTRICTION ENDONUCLEASE ANALYSIS

The CMV isolates analyzed by restriction endonuclease analysis using ethidium bromide staining were propagated in three HNFF cell monolayers contained in 175 cm^2 Falcon flasks. To obtain the most possible DNA, the DNA isolation procedure used the HNFF cell monolayers when 90 to 95% of the cell monolayer showed CPE. At this point the medium was discarded. The monolayer was washed with PBS which was then removed. A 2 ml volume of HIRT Buffer with 1 mg/ml Protease (Sigma Chemical Company) was distributed among three Blake bottles. After the salt precipitation of the host cell DNA (described previously), the supernatant was collected,

incubated with 1 mg/ml Protease for approximately 3 hours at 37°C after which the phenol and ether extractions were performed. RNase treatment was not included. The protease treatment at the initiation of the procedure would deactivate this enzyme and, due to the small sample size, successive DNA precipitations to remove protease may have further reduced the amount of DNA isolated. Following dialysis the sample was diluted 1:2 with cold ethanol with 1/10 volume 3M sodium acetate in a 15 ml centrifuge tube (Corex tube, Corning). The solution was placed in a -20°C freezer overnight then centrifuged at 4°C for at least 30 minutes at 15,000 rpm (30,000 g) in a refrigerated B-20 centrifuge (I.E.C.). The supernatant was decanted, then the centrifuge tube was inverted to air dry for at least one day. A total volume of 1 ml TRIS-EDTA buffer (TE) (Appendix III) was used to suspend the DNA precipitated in the centrifuge tube. The sample was placed at 37°C to facilitate the suspension of the DNA in the TE buffer. The sample was stored in a 1.5 ml Eppendorff tube at 4°C until required. The concentration of DNA contained in the sample was determined by the ratio of the spectrophotometric analysis at the wavelengths 260 nm and 280 nm (Maniatis et al 1982). A sample of 2 ul of the sample was diluted in 998 ul TE buffer for this analysis.

The volume of viral DNA to be analyzed in the comparative gels was determined by using various sample

volumes to determine which volume produced the best electrophoretic fragment pattern after restriction endonuclease digestion. Aliquots from 10 ul to 87 ul of sample in Eppendorff tubes were incubated with 10 ul of the appropriate 10X concentrated enzyme buffer (Appendix III), 5 ul of restriction enzyme and deionized water to make the volume to 100 ul. Bacteriophage lambda DNA (200 ng to 400 ng) (Sigma Chemical Co.) was used as a control to ensure that the restriction endonuclease procedure was effective. Bacteriophage lambda DNA electrophoretic fragment patterns also served as markers against which the the CMV DNA fragment bands were compared. An 0.8% agarose gel in TPE buffer was run at a voltage adjusted to 55 V for the first 1/2 hour then lowered to 35 V overnight (16-16 1/2 hours). The buffer used during the electrophoresis was TPE (Appendix III).

Following electrophoresis the gel was stained with a 0.6% solution of ethidium bromide in the dark for one hour. The gel was then rinsed with running water for 1/2 hour to remove the excess stain. The DNA bands were visualized by a UV transilluminator (Fotodyne) at 330 nm and photographed with Kodak Professional Contrast Process Pan Film #4155 (Eastman Kodak Company) for a permanent record.

The volume of CMV sample to be analyzed by restriction endonucleases was determined by both the DNA concentration

analysis by spectrophotometry, and also from the information obtained from the original gel (in which varying volumes of the sample DNA were used). A standard gel format was employed so that comparisons of CMV DNA restriction patterns from different gels could be made. The gel format consisted of a Bacteriophage lambda DNA control (200 ng to 400 ng), a CMV control [15 ug of day care centre isolate 100 (DC 100)] and up to seven CMV isolates.

The electrophoretic fragment patterns of the day care centre CMV isolates were compared to determine if epidemiological relationships existed between the isolates. Usually the photographic negatives themselves, which recorded the electrophoretic fragment patterns for the virus samples run on an individual agarose gel, were used in the comparison rather than the photographic prints. Better resolution of the electrophoretic fragment patterns was found with the negatives than that with the photographic prints produced from these negatives. The Bacteriophage lambda and/or CMV controls were used as standard markers for reference points against which the electrophoretic fragment patterns of the CMV isolates were compared. The large number of CMV isolates outnumbered the well spaces on an individual gel. CMV restriction fragment patterns from many gels would have to be compared. Therefore, by using the Bacteriophage lambda DNA and CMV controls, comparisons could be made among the electrophoretic fragment patterns analyzed

on different agarose gels to determine whether transmission of the virus had occurred amongst the observed population.

The photographic prints exhibited in this thesis consist of composites made from the photographs of the electrophoretic fragment patterns in the agarose gels. Because individual DNA isolates varied considerably in ethidium stained band intensities, the gel was photographed at various exposures to ensure that all lanes containing DNA fragment patterns were visible and the best prints of each lane were put together to make the composite. Composite photographs of electrophoretic fragment patterns from different gels have also been used by Adler (1985A, 1988).

To compensate for the electrophoretic fragment pattern length differences between the gels, the Bacteriophage lambda banding pattern was used to standardize the patterns. There were considerably fewer bands produced from Bacteriophage lambda than with the CMV control. The identification of reference bands was easier if there were only a few bands. When the negative was printed, the distance between the well origin and a reference Bacteriophage lambda DNA band for every analysis with the particular enzyme were equidistant. The lambda control and the CMV control were only printed once on a composite or in some cases were omitted. The clearest photograph for each CMV isolate's electrophoretic fragment pattern for an enzyme

analysis were placed together and rephotographed to provide the final composite of the gel with the electrophoretic patterns of the DNA fragments as distinct as possible.

In many of the composite prints the electrophoretic fragment patterns of the CMV isolates were from restriction endonuclease digests analyzed in the same gel. However, due to the varying intensities of the patterns, each electrophoretic fragment pattern was printed individually in order that the final composite photograph would consist of patterns with similar intensity. The varying pattern intensities may have been due to pipetting inaccuracies and/or incomplete mixing of the DNA samples. Even by adjusting the photographs of each individual electrophoretic fragment pattern to reflect similar intensities, many of the photographs do not exhibit the clarity of the negatives. Numerous attempts were undertaken to produce the clearest electrophoretic fragment patterns possible from each negative. Both contact and enlarged prints were produced in order to produce a clear photograph of the patterns. In only a few cases was it possible to obtain a print which exhibited the clarity of electrophoretic fragment pattern on the negative.

E. STATISTICAL ANALYSIS

The research design chosen for this study to estimate the CMV urine excretion prevalence in various Halifax day care centre populations was a retrospective cross-sectional survey. In the analysis the chi-square test, the Mann-Whitney rank sum test and the Kruskal-Wallis statistic were used to assess whether differences in the day care centre populations existed and to determine whether risk factors could be identified for the excretion of CMV by the day care centre children.

In order for comparisons to be made among the day care centre populations, the demographic descriptors for the populations had to be identified. The day care centres surveyed were grouped into different categories according to the Nova Scotia Department of Social Services' classification system (as previously described). For the statistical analysis of the day care centres descriptors, the centres were classified into two groups, (1) Registered versus Private, and (2) all day care centres in which urine samples were collected. The population from day care centre F, which was not surveyed by urine collection, was omitted from the second analysis. Because the CMV urine excretion prevalence data would be compiled to determine an overall CMV excretion prevalence for the five day care centres

surveyed, the Registered and Private day care centres would have to be compared to determine whether differences could be identified between the two groups.

1. QUESTIONNAIRE

The parents of the day care centre children were sent a questionnaire via the day care centre staff to identify the descriptors for their child (age, sex, and number of siblings) as well as descriptors relating to the child's family [parental education and occupation (socioeconomic factors) and parental age]. The questionnaires were then returned to the day care centre. Unfortunately the parents did not always complete the forms, usually all questions regarding the child were answered; however, the parental descriptors in some cases were omitted.

The attributes used in the statistical analysis of the day care centre survey data included:

A - child's age classified into four categories. These categories were less than 18 months, from 18 months to 31 months, from 32 months to 41 months, and from 42 months to 60 months.

B - child's sex.

C - number of siblings classified into four categories. These categories were none, one sibling, two siblings, and more than two siblings.

D - parental age classified into four categories. These categories were from 20 to 24 years, from 25 to 29 years, from 30 to 34 years, and 35 years and over.

E - parental education classified into three categories. These categories were high school education, post-high school education and graduate school education.

F - parental professions; classified by Backstrom and Hursch-Cesar (1981) and condensed into four categories (Table 5). These categories were unemployed, nonprofessional, professional, and student.

2. ANALYSIS

Hypotheses were formulated for each of the descriptors to determine whether the descriptor was independent of the type of day care centre or between day care centres. Statistical analyses of the data included the chi-square test, the Mann-Whitney rank sum test and the Kruskal-Wallis statistic. The test employed depended on whether the attribute being examined was unranked, for example child's sex (chi-square test), ranked with two samples, for example child's age in the Registered versus Private day care centres (Mann-Whitney rank sum test) or ranked with three or more samples, for example child's age in the three Registered day care centres (Kruskal-Wallis statistic).

The chi-square test determines whether two or more

TABLE 5 Classification of parental occupations.

The occupations of the day care centre children's parents were categorized into four groupings, modified from Backstrom and Hursch-Cesar (1981).

<u>OCCUPATIONAL GROUPING</u>	<u>DESCRIPTION</u>
UNEMPLOYED	PARENTS NOT CURRENTLY WORKING, HOUSEPARENTS
NONPROFESSIONAL	CLERIAL, SALES WORKERS, CRAFTSMEN, OPERATIVES, SERVICE MANAGERS, LABOURERS FARMERS AND THEIR KINDRED WORKER
PROFESSIONAL	PROFESSIONAL, TECHNICAL, AND KINDRED WORKERS
STUDENT	POST SECONDARY EDUCATION STUDENTS

attributes are independent by hypothesis testing. Two hypotheses are formulated. The alternate hypothesis (H_a) which assumes the variables being tested are dependent and the null hypothesis (H_0) which assumes the variables are independent. The amount of deviation between the actual results and calculated expected results is measured then based on the value obtained from this measurement, the null hypothesis is either accepted or rejected. In order for a chi square test to be valid each of the expected values must be at least 5 (Marks 1982).

The Mann-Whitney test is used to compare two populations with independent samples. It also tests the null hypothesis. In this test the measurements are ranked according to their magnitude. The results are compared with the distribution of all possible rank sums to determine whether the pattern of ranking is compatible with the null hypothesis.

The Kruskal-Wallis Statistic tests whether differences exist among several means. The test is a generalization of the Mann-Whitney rank sum test but three or more observation data sets are being compared. Again each observation is ranked and the measurement of the average ranks within each group is compared to determine the deviation from the average rank of all the observations. In all the statistical analyses performed; a P value of less than or

equal to .05 was considered to be a significant difference.

The attribute ranking in the Mann-Whitney rank sum test and the Kruskal-Wallis statistic was based on assigning the lowest category a rank of one and increasing by one ordinal as the rank increased, for example the attribute describing the parent's education. Those parents with only high school education were assigned a rank of one, parents with post-high school education were assigned the rank of two and parents who had attended graduate school were assigned the rank of three.

3. CYTOMEGALOVIRUS EXCRETION RISK FACTOR ANALYSIS

The statistical analysis to determine the risk factors correlating with excretion of CMV by day care centre children involved classifying the day care centres into two categories; the first grouping consisted of all day care centres surveyed including all information gathered during the survey period from day care centre A and the second grouping used the data obtained from the three year survey of day care centre A. The responses for children excreting CMV and children not excreting the virus were compared. The data from the initial virus sampling for children excreting the virus was used in the analyses. Hypotheses were then formulated for the demographic descriptors to determine whether the descriptors were independent of CMV excretion in

the day care centre children. Statistical analyses were performed by the above mentioned tests.

4. POWER

The power of a test is a measurement of the ability of that test to reject the null hypothesis when, in fact, the null hypothesis is false. The power is related to the significance criterion, the sample size and the effect size. Given the sample size, significance criterion and the effect size, the power of the test may be determined.

The size of the effect for chi square testing is categorized into small, medium and large. The value for the effect size is determined by an estimate from previous studies reported in the literature. For the CMV urine excretion survey, based on the previous studies, a medium size effect was used.

III. RESULTS

The results of the CMV excretion prevalence and transmission study within Halifax, Nova Scotia day care centre children are described in four sections; A - definition of the day care centre populations, B - CMV excretion prevalence in day care centre children, C - definition of risk factors associated with CMV excretion, and D - the molecular epidemiology of the CMV isolates.

A. DEFINITION OF DAY CARE CENTRE POPULATIONS

As previously stated during the year 1984 approximately one-quarter of all Halifax children under the age of six years attended licensed day care centres. To study CMV excretion prevalence and transmission within Halifax day care centre populations, the selection of a subset of Halifax day care centres for the collection of urine from the children in attendance was required. The description of the individual day care centres and divisions of day care centres (or to determine the possible risk factors associated with CMV excretion within the day care centre children as discussed later) required statistical analyses to define the demographic descriptors of the population (children attending day care). Statistical analysis of demographic descriptors for the day care centre populations

would identify whether significant differences among the populations existed. One objective of this study was determining an overall CMV excretion prevalence for children attending Halifax day care centres. This calculation exploited the assumption that the day care centre populations were similar in most of the demographic descriptors.

To survey all the children attending all the Halifax day care centres was not feasible due to the number of children, technical facilities available, and time required to undertake a survey of such magnitude. Therefore a subset of six day care centres was selected to represent the different day care centre enrollment size ranges. As previously stated the enrollment in registered day care centres tended to be larger than that seen in the private centres. The number of children aged from 12 months to six years in each of the three registered day care centres ranged from 25 to 76, whereas the number of children, aged between one and a half and six years, in the private day care centres ranged from 17 to 50 (Table 6).

The omission of day care centre F from the CMV prevalence survey was due to hostility of the parents to the study. The survey form data for this day care centre was only used in the statistical analyses to determine whether differences existed between the chosen subsets of registered

TABLE 6 Tabulated responses for each day care centre to the survey letter.

The Halifax day care centres selected to be examined for presence of CMV urine excretion in children were first surveyed by questionnaire to determine the numbers of parents willing to allow their child to participate in the CMV urine excretion prevalence survey and the demographic descriptors of the populations. The survey response has been categorized into registered and private day care centres.

DAY CARE CENTRE	CLASSIFICATION	AGE RANGE (Y)	NO. CHILDREN	NO. RESPONSES	NO. POSITIVE RESPONSES
A	REGISTERED	1-6	76	55 (46%)	32 (91%)
B	REGISTERED	1-6	45	25 (56%)	24 (96%)
C	REGISTERED	2-6	25	21 (84%)	21 (100%)
D	PRIVATE	3-6	28	21 (75%)	17 (81%)
E	PRIVATE	1.5-6	17	8 (47%)	8 (47%)
F	PRIVATE	3-6	50	45 (90%)	35 (78%)
TOTALS			241	155 (64%)	137 (57%)

Y - YEARS
NO. - NUMBER

and private day care centres.

Two distinct organizational formats were identified amongst the day care centres examined. The first composition consisted of distinct care areas for the different age grouping (Day care centres A, B and C) and the second organization cared for children of all ages in a common area (Day care centres D, E and F). In both formats, however, the children could congregate with other children of different ages in common playareas. Close personal contact among children within the day care centre occurred; thus, organisms spread by close personal contact could be transmitted among the day care centre children.

The demographic descriptor analysis utilized the data collected from the originally selected six day care centres. The survey and consent forms sent to the parents during at least two mailings, when possible, elicited 155 responses representing 64% of the day care children (Table 6). The individual day care centre responses varied from 46% to 90%. The parents of children attending the private day care centres (79%) were more likely to return the survey forms than the parents of the children attending registered day care centres (56%), statistically significant by chi-square, $p < .001$. However, the probability of the parents of the children attending the registered day care centres to allow their children to participate in the survey was higher

(95%), as compared to the parents of the private day care centre children (81%), statistically significant by chi-square, $p < .01$. The power of these statistical tests to detect a medium sized effect between the two classifications of day care centres would be high (98), so trends in these analyses should be detected.

A general description of the Halifax day care centre populations participating in this survey was; mean age of the children was 3 years of age, mean ages of the parents were 29 years of age for the mother and 32 years of age for the father, and most of the day care centre children did not have siblings (Table 7). The majority of the parents were either university students or university graduates. The parental occupations reflected the parental education, most parents were either university students or professionals. On the basis of education and occupation, the six day care centres reflected a middle socioeconomic clientele.

The demographic data returned on the survey forms, in some cases, had omissions in the information volunteered by the children's parents. Some of the parents would selectively answer the questions, omitting any information they did not want included in the survey. Most of the forms contained all the information pertaining to the child; however, variations in information content occurred with the parental demographic descriptors. Tables 7 reflected this

TABLE 7 Demographic descriptors of the surveyed Halifax day care centres.

Attributes obtained from data collected from the parents of the children attending all surveyed Halifax day care centres. Day care centre F data is presented in this chart, although, the data was only used in the comparison of registered and private day care centres. The data contained in each descriptor may not total the numbers of parental responses described Table 6. The parents did not consistently answer all the questions contained on the survey forms.

	DAY CARE CENTRE A	DAY CARE CENTRE B	DAY CARE CENTRE C	DAY CARE CENTRE D	DAY CARE CENTRE E	DAY CARE CENTRE F
DEMOGRAPHIC CHARACTERISTICS						
CHILD'S SEX						
MALE	19	12	10	4	3	17
FEMALE	13	13	11	13	5	17
AGE GROUP IN YEARS						
<1 1/2	5	0	0	0	0	0
1 1/2 < 2 1/2	9	8	6	0	2	0
2 1/2 < 3 1/2	7	5	7	4	6	12
3 1/2 < 6	11	17	8	13	0	11
NUMBER OF SIBLINGS						
0	19	23	8	8	5	5
1	9	6	9	9	2	14
2	3	0	2	0	0	8
>2	0	0	0	0	0	2
PARENTAL AGE IN YEARS						
(MOTHER)						
20 - 24	3	1	1	0	0	0
25 - 29	13	9	12	2	6	3
30 - 34	12	11	4	7	0	13
35+	1	6	0	8	0	15
(FATHER)						
20 - 24	2	0	0	0	0	0
25 - 29	7	4	7	1	3	3
30 - 34	7	9	4	5	2	9
35+	9	10	1	10	1	17
PARENTAL EDUCATION						
(MOTHER)						
HIGH SCHOOL	6	6	6	1	2	0
POST HIGH SCHOOL	18	11	6	13	4	24
GRADUATE SCHOOL	0	9	2	3	1	7

Table 7 Demographic descriptors of the surveyed Halifax day care centres (continued).

(FATHER)						
HIGH SCHOOL	4	2	5	1	4	0
POST HIGH SCHOOL	14	10	10	10	2	18
GRADUATE SCHOOL	5	8	2	5	1	12
PARENTAL OCCUPATION						
(MOTHER)						
UNEMPLOYED	1	0	0	1	0	11
NONPROFESSIONAL	8	8	10	4	5	0
PROFESSIONAL	12	14	3	10	2	18
STUDENT	5	5	2	2	0	2
(FATHER)						
UNEMPLOYED	1	0	1	1	0	0
NONPROFESSIONAL	9	1	8	4	6	3
PROFESSIONAL	10	17	4	11	0	26
STUDENT	5	3	2	0	1	1

lack of information for all demographic descriptors; therefore, the numbers do not always total to give the complete number of survey participants.

When the survey data from the day care centres both registered versus private and all centres surveyed for prevalence of CMV (demographic data from day care centre F omitted) were analyzed only a few of the descriptors were found to differ significantly, child and parental age and number of siblings (Table 8). Day care centre D was found to have children with older parents. The socioeconomic demographic descriptors, parental education and occupation, were found to be similar both in the registered versus private analyses and amongst the five day care centres surveyed for presence of CMV. The power of the statistical analysis to detect trends in these analyses was quite high, most tests were around 75. A visual examination of the survey data for the presence of trends within the attributes confirmed the results obtained by statistical analysis. No trends other than those found by statistical analysis were detected among the demographic descriptors. Therefore, the CMV excretion prevalence data could be compiled to calculate an overall CMV excretion prevalence for the selected Halifax day care centres.

TABLE 8 Analysis of the demographic attributes for the surveyed Halifax day care centres.

The registered and private day care centres were compared to determine whether major differences between the two categories of Halifax day care centres could be identified. The second analysis compared the demographic data for all the day care centres used in the CMV excretion prevalence survey.

DEMOGRAPHIC ATTRIBUTES	REGISTERED VS PRIVATE DCC'S	ALL DCC'S SURVEYED FOR CMV EXCRETION
CHILD'S SEX	ns (1)	ns (1)
MALE		
FEMALE		
AGE GROUP IN YEARS AND RANK	ss (2)	ns (3)
1. <1 1/2		
2. 1 1/2 < 2 1/2		
3. 2 1/2 < 3 1/2		
4. 3 1/2 < 6		
NUMBER OF SIBLINGS AND RANK	ss (2)	ns (3)
1. 0		
2. 1		
3. 2		
4. >2		
PARENTAL AGE IN YEARS AND RANK (MOTHER)	ss (2)	ss (3)
1. 20 - 24		
2. 25 - 29		
3. 30 - 34		
4. 35+		
(FATHER)	ss (2)	ss (3)
1. 20 - 24		
2. 25 - 29		
3. 30 - 34		
4. 35+		
PARENTAL EDUCATION AND RANK (MOTHER)	ns (2)	ns (3)
1. HIGH SCHOOL		
2. POST HIGH SCHOOL		
3. GRADUATE SCHOOL		

TABLE 8 Analysis of the demographic attributes for the surveyed Halifax day care centres (continued).

(FATHER)	ns (2)	ns (3)
1. HIGH SCHOOL		
2. POST HIGH SCHOOL		
3. GRADUATE SCHOOL		

PARENTAL PROFESSION

AND RANK

(MOTHER)	ns (2)	ns (3)
1. UNEMPLOYED		
2. NONPROFESSIONAL		
3. PROFESSIONAL		
4. STUDENT		

(FATHER)	ns (2)	ns (3)
1. UNEMPLOYED		
2. NONPROFESSIONAL		
3. PROFESSIONAL		
4. STUDENT		

DCC'S - DAY CARE CENTERS

ss - STATISTICALLY SIGNIFICANT $P > .05$

ns - STATISTICALLY NOT SIGNIFICANT

(1) - CHI-SQUARE TEST (DETERMINES WHETHER DIFFERENCES ARE INDEPENDENT)

(2) - MANN-WHITNEY TEST (DETERMINES WHETHER TWO POPULATIONS WITH RANKED ATTRIBUTES ARE DIFFERENT)

(3) - KRUSKAL-WALLIS STATISTIC (DETERMINES WHETHER DIFFERENCES EXIST AMONG SEVERAL MEANS WHEN RANKED ATTRIBUTES ARE ANALYZED)

**B. CYTOMEGALOVIRUS PREVALENCE IN HALIFAX DAY CARE
CENTRE CHILDREN**

Children with asymptomatic CMV infections may excrete the virus in their urine over extended periods of time (Weller et al 1957). Although there are considerable difficulties with isolating CMV from children, urine isolation collection was selected as the method to determine CMV prevalence. The basis of this decision included the fact that urine collection consists of a noninvasive technique for the isolation of the virus. Children currently excreting the virus are detected and the virus can then be identified by restriction endonuclease analysis of the viral genome. A CMV antibody survey would have detected children with a previous, as well as a current CMV infection; however, the procedure is invasive. Identification of virus excretion and restriction endonuclease analysis to determine the transmission data would not have been possible to determine if a CMV antibody survey was utilized because the virus is not isolated. It was also anticipated that the ethics committees and the parents of the children attending the day care centres would not allow an invasive technique such as venous puncture to be performed on the children.

Although saliva excretion has also been used to determine CMV excretion presence, the ability of this method

to detect CMV is not as accurate as urine isolation of CMV (Hutto et al 1985B). CMV excretion in saliva has been shown to occur with less frequency than CMV excretion in urine. Therefore a child may be excreting the virus in its urine but not in the saliva. The number of children identified as being infected with CMV as determined by saliva excretion would be considerably less than that determined by urine excretion isolation (Hutto et al 1985B). Also the duration of time that the virus was shed by day care children was found to be longer for urine excretion than saliva. Supplemental saliva isolation method was not employed with the urine collection. Such a combination may have improved the child compliance; however, the results of such a combination would not be accurate in the case of a child sampled only by saliva collection.

CMV infections may be detected via other techniques which include direct examination of the specimens, fluorescein-conjugated monoclonal antibody staining procedures, electron microscopy, radiolabelled DNA probes, etc. These techniques were not employed to assist in identifying children with CMV infections. This survey attempted to keep as many variables, such as CMV identification methods, constant with the previously reported literature concerning presence of CMV in day care centre children. The children's urine specimens were also not concentrated to increase the possibility of detecting

CMV or increasing the yield of virus. The urine specimens collected from the children were not the ideal clean-catch mid-stream urine specimen, but rather any urine which could be obtained. Therefore, bacterial contamination was a major factor in isolating CMV from these specimens. If the urine specimens were concentrated, the possibility existed of concentrating any contaminating normal flora.

As previously stated the children's urine samples were processed for the identification of CMV presence by the foreskin fibroblast system of cell culture (Embil and Faulkner 1964). The foreskin system of identifying and propagating CMV was employed for a number of reasons, which include the human origin of the cells due to the species-specific nature of CMV, the availability of the tissue, and the convenience of identification of the virus. A tentative identification could be made based on the CPE produced by the virus in the HNFF cell monolayer, which was then confirmed by restriction endonuclease analysis of the viral genome. Only one virus isolate was misidentified. An adenovirus was found to produce a similar CPE in the HNFF cell monolayer to the CPE produced by CMV isolates.

The isolation time between inoculating the specimen on the cell monolayers in the cell culture tubes and the first signs of CMV CPE is usually four weeks. At the onset of this study six weeks was the incubation time period

allotment prior to a negative determination for virus presence. Since the first few CMV positive specimens were found to require approximately five weeks before visible signs of CPE were evident, the specimens were assessed for at least eight weeks prior to making the decision that CMV was not present. The extended time period was assumed to be due to a low CMV concentration in the children's urine (Eizuru et al 1984); however, this assumption was not confirmed.

1. VIRUS ISOLATION DURING THE CYTOMEGALOVIRUS EXCRETION PREVALENCE SURVEY

As previously described, day care centre F was omitted from the virus survey of day care centre children due to the negative attitude displayed by the parents. Interestingly, the population of day care centre F consisted of older parents with more than one child, indicative of well-established families. This may have influenced the attitude towards the survey.

Of the original child participants in the CMV excretion prevalence survey, 106 children from day care centres A to E, urines were obtained from a total of 75 children. The remaining 31 children had either refused to cooperate or left the day care centres during the time period between the survey letter distribution and the collection of urine

specimens from the children. As previously stated if a child refused to cooperate three times, the child was then dropped from the sampling of the day care centre population. The compliance of the children to produce urine samples for the five day care centres was 72%, ranging from 56% to 80% (Table 9).

The compliance of the children, in part, seemed to depend on the attitude of the day care centre staff towards the study. Both Ethics committees required that the day care centre staff collect the urine specimens from the children. The staff from day care centre C was exceedingly positive in attitude towards the survey, even using games to induce the children to provide samples. As a result this day care centre had the highest percentage of children providing samples.

Another factor which influenced the compliance of the children was the presence of a vocal noncompliant child within the day care centre group being sampled. During the 1984 survey of day care centre A one such child attended the "senior" group. A rebellious attitude towards providing a specimen quickly swept through the senior group of children. Although separating this group of children into smaller units prior to approaching the children for samples increased the probability of obtaining the individual child's cooperation, several children within this group

still refused to provide specimens.

As previously described the initial determination of CMV presence in a urine specimen was based on the presence of CMV-like CPE in HNFF cell monolayers; confirmation of the virus's identity was based on the restriction endonuclease analysis of the viral genome. Ten day care centre urine specimens were found to produce CMV-like CPE in HNFF cell monolayers. CMV was isolated from four children attending day care centre A, and two children attending each of day care centres C, D and E. In addition, an adenovirus was found to be excreted by a child attending day care centre A. The CMV excretion prevalence for the individual day care centres, thus, ranged from 0% to 33% with an overall excretion prevalence of 13% (Table 9). The 95 percent confidence interval for the overall CMV prevalence is from 6% to 21%. The two centres with CMV prevalences of 0% and 33% have large 95% confidence intervals, 0-23% and 0-70% respectively; therefore, these CMV prevalences were found not to be statistically significant from the remaining day care centre CMV prevalences. The number of children required to be surveyed in order to estimate the CMV urine excretion prevalence (estimated to be low) within 8% with a 95% confidence coefficient would be 77 children.

Virus shedding within the age groupings of the day care centre children was found to be similar among the three older age groupings; toddler, junior and senior (Table 10). The

TABLE 9 CMV urine excretion shedding in Halifax day care centre children participating in the survey.

Five of the six Halifax day care centres selected for this study were surveyed for the presence of urinary CMV excretion. Unfortunately some of the children either refused to provide a urine sample or were not longer attending the day care centre when the samples were obtained. The sample population consisted primarily of children from middle socioeconomic families.

DAY CARE CENTRE	NO. CHILDREN PARTICIPATING	NO. CHILDREN SURVEYED	NO. CHILDREN EXCRETING CMV	STANDARD* DEVIATION	95% CONFIDENCE INTERVAL
A	35	26 (74%)	4 (15%)	0.07	2% - 28%
B	25	14 (56%)	0 (0%)	0.06	0% - 23%
C	21	17 (80%)	2 (12%)	0.08	0% - 27%
D	17	12 (71%)	2 (17%)	0.11	0% - 38%
E	8	6 (75%)	2 (33%)	0.19	0% - 70%
OVERALL	106	75 (72%)	10 (13%)	0.04	6% - 21%

* THE POPULATION PROPORTION STANDARD DEVIATION CAN BE REFERRED TO AS THE STANDARD ERROR OF THE POPULATION AND IS NOT REPRESENTED AS A PERCENTAGE.

TABLE 10 CMV excretion prevalence in the different age groupings of the surveyed Halifax day care centre children.

In some cases multiple specimens were analyzed for an individual child over the study period. Because children can be infected with CMV at any age, each negative isolation was recorded as a separate data point. Thus, the number of specimens do not coincide with the actual number of children participating in the study. The initial data for children found to be excreting the virus was used to identify the age at which excretion began.

AGE GROUP IN MONTHS	All CHILDREN SURVEYED	STANDARD* DEVIATION	95% CONFIDENCE INTERVAL	DAY CARE CENTRE A	STANDARD* DEVIATION	95% CONFIDENCE INTERVAL
UNDER 18	13% (1/8)	0.11	0% - 35%	13% (1/8)	0.11	0% - 35%
18 - 31	11% (5/47)	0.04	3% - 19%	11% (4/35)	0.05	1% - 21%
32 - 41	18% (7/38)	0.06	8% - 28%	22% (5/23)	0.08	6% - 38%
42 - 60	11% (6/54)	0.04	3% - 19%	9% (3/32)	0.05	0% - 18%

* THE POPULATION PROPORTION STANDARD DEVIATION CAN BE REFERRED TO AS THE STANDARD ERROR OF THE POPULATION AND IS NOT REPRESENTED AS A PERCENTAGE.

highest CMV excretion frequencies were found in the junior group; children aged 32 to 41 months. These results were also reflected in the repeated day care centre A surveys. The CMV excretion prevalences increased from under one year to approximately 32 to 41 months, then a decline occurred. It must be noted that these observations were based on small sample sizes.

The overall CMV excretion prevalence for the Halifax day care centre children populations was determined by combining the data from the individual day care centres. The five Halifax day care centres surveyed for CMV excretion prevalence reflected a mostly professional population with post secondary education, indicative of middle socioeconomic status. However, since day care centres in the Halifax area are not divided into low, middle and upper socioeconomic status day care centres (only into registered and private) and few demographic descriptor differences existed between the divisions of day care centres (parental occupation was found to be similar), it can be concluded that most Halifax day care centres would also reflect a middle socioeconomic status population (parents with post high school education and at least one parent having a professional occupation). Because there were no statistically significant differences in the CMV excretion prevalences for the individual day care centres, the compilation of the data should be a valid estimate of the excretion prevalence of CMV for children attending Halifax day care centres.

2. VIRUS ISOLATION FROM DAY CARE CENTRE A

Limited data for CMV transmission among day care centre populations have been determined from one sampling of a day care centre (Adler 1985A). The children excreting CMV isolates found to have identical electrophoretic fragment patterns by restriction endonuclease analysis of the virus genomes can be said to have an epidemiological relationship; that is, the virus has been transmitted either directly or indirectly from one child to another. However in such a case, the progress of transmission - which child actually transmitted the virus to the other children - cannot be defined. In order to describe the chains of CMV transmission among the day care centre children, multiple surveys over extended periods of time must be performed to determine the identity of the children excreting virus and which children are not currently infected. Previously uninfected children found to commence virus excretion may be identified as recently infected.

The children attending day care centre A were studied longitudinally to determine if CMV transmission occurred within the day care centre environment. The choice of the day care centre was based on the large number of children attending the centre and the staff's cooperative attitude regarding the survey. The day care centre population was sampled three times, December 1984, June 1985 and December

1986. Intervals of at least six months were selected so that the previously nonexcreting children which had become newly infected by CMV would begin to excrete virus. Other day care centres surveys also used intervals of at least six months (Murph et al 1986). The third survey of the day care centre occurred at an interval greater than six months to allow transmitted CMV infections time to manifest. During that time period surveys were performed on all the other day care centre populations.

The parental responses to the survey forms showed an increased number of parents with each successive survey (Table 11). The parent's exposure to multiple letters describing the survey was thought to have influenced the parents response. During each survey, repeat letters were sent to all nonresponding parents. Throughout the successive surveys all parents of children who did not have previous consent to participate were sent further letters in anticipation of obtaining consent. The compliance of the children to provide urine samples, however, remained approximately the same during the three surveys (Table 11).

During the three surveys from 1984 to 1986, a total of 13 children were found to be excreting CMV (Table 12). In 1984, four children were found to be excreting CMV, the original four children plus six additional children were determined to be excreting CMV in the 1985 survey and in

TABLE 11 Day care center A CMV urine excretion survey data.

Day care centre A was surveyed a total of three times over an approximately two year period. The survey results were compiled for these surveys.

	DECEMBER 1984	JUNE 1985	DECEMBER 1986
DAY CARE CENTRE ENROLLMENT	72	72	68
PARENTAL RESPONSE	35 (49%)	48 (67%)	50 (74%)
POSITIVE RESPONSE	32 (91%)	46 (96%)	47 (94%)
CHILD COMPLIANCY	26 (81%)	38 (83%)	42 (89%)
CMV URINE EXCRETION	4 (15%)	10 (26%)	6 (14%)

TABLE 12 Description of the urine specimens obtained from day care centre A.

A total of 106 urine specimens were collected from the children attending day care centre A during the three surveys. Of the children surveyed, 13 children were found to excrete CMV for a total of 20 CMV positive urine samples. Also one child (DC 23) was identified to be excreting an adenovirus-like virus during the 1984 survey. The virus was not able to be reisolated in any future specimens. Each specimen number refers to an individual child.

SPECIMEN NUMBER	DECEMBER 1984	JUNE 1985	DECEMBER 1986
01	+	+	C
02	-	-	-
03	-	+	C
04	-	C	C
05	-	-	C
06	-	-	C
07	-	-	-
08	-	-	-
09	-	-	C
10	-	-	C
11	-	-	C
12	-	C	C
13	-	+	C
14	+	+	-
15	+	+	+
16	-	-	-
17	-	-	C
18	-	-	C
19	-	C	C
20	-	+	C
21	+	+	+
22	-	+	C
23	†*	-	C
24	-	C	C
25	-	C	C
26	-	-	C
27	A	-	-
28	A	-	C
29	C	-	-
30	A	+	C
31	C	-	-
32	C	-	-
33	A	-	+

+ CMV PRESENT

†* ADENO-LIKE VIRUS PRESENT

- CMV NOT ISOLATED

A - PARENTS REFUSED PERMISSION FOR THAT URINE COLLECTION

B - CHILD REFUSED TO COOPERATE

C - CHILD NOT ATTENDING THE DAY CARE CENTER DURING THE URINE COLLECTION PROCEDURE

TABLE 12 Description of the urine specimens obtained from day care centre A (continued).

34	A	-	C
35	A	-	C
36	A	-	C
37	A	+	+
38	B	-	C
39	B	-	C
40	B	-	C
41	B	-	C
42	B	-	C
43	B	-	C
93	C	C	-
94	C	C	-
95	C	C	-
96	C	C	-
97	C	C	-
98	C	C	-
99	C	C	-
100	C	C	+
101	C	C	-
102	C	C	-
103	C	C	-
104	C	C	-
105	C	C	+
106	C	C	-
107	C	C	-
108	C	C	-
109	C	C	-
110	C	C	-
111	C	C	-
112	C	C	-
113	C	C	-
114	C	C	-
115	C	C	-
116	C	C	-
117	C	C	-
118	C	C	-
119	C	C	-
120	C	C	-
121	C	C	-

1986 six children, three previously positive children plus three additional children, excreted CMV. The 1985 study showed an increase in CMV virus excretion from 15% to 26%; however, during the 1986 survey the CMV prevalence decreased to the original prevalence of 15%. Statistical analysis by chi-square found the differences in number of children excreting virus for the surveys to not be significant. By the time of the 1986 survey many of the children from the 1984 survey had progressed from the day care centres to school or had been moved from the day care centre. Day care centre populations are very mobile and the population of this day care centre was found to be representative of this mobility.

As previously stated, all the children positive for CMV excretion in the first survey were also positive during the second survey. Of the four children positive in the June 1985 survey and still attending the day care centre during the third survey, December 1986, three children were still excreting CMV. Throughout the three surveys only one child (DC 14) was found to have stopped excreting CMV. When this previously CMV-excreting child (1984 and 1985) was assessed for CMV excretion during the third survey in 1986, the child was no longer excreting CMV. The other four CMV-excreting children, who had multiple urine specimens examined for presence of CMV, remained CMV positive during the repeat

sampling.

**C. STATISTICAL ANALYSIS OF RISK FACTORS FOR CYTOMEGALOVIRUS
EXCRETION**

During reported CMV excretion prevalence surveys comparing day care centre children against control children, the only risk factor to be identified for increased CMV infection is attendance in a day care centre (Pass et al 1982, 1984). The day care centre population surveyed in this study cannot be said to be representative of the general Halifax population. However, this population was representative of children from middle to upper socioeconomic families from the Halifax area.

The demographic data collected, via the survey forms distributed to the parents of the children attending the day care centres surveyed, was analyzed to determine whether CMV excretion could be correlated with previously described risk factors for CMV infection; for example age or socioeconomic status of the children's families. From the survey forms two groupings of data were used to determine if risk factors existed for CMV excretion by the day care centre children. As previously mentioned, due to the inconsistent completion of the survey forms, the demographic descriptor numbers may not all total to the same number. The information was classified into (1) the results from all the children collected throughout the project and (2) the results from the children attending day care centre A.

1. ALL DAY CARE CENTRES

All children surveyed for the presence of CMV in urine were statistically analyzed to determine whether risk factors for CMV infection within children attending day care centres could be identified. Included in this analysis were the children from the multiple surveys of day care centre A. Data descriptions are found in Table 13. Data for the children excreting CMV was compared against the data for the children not excreting the virus. No risk factors for CMV excretion in the day care centre children could be identified.

2. DAY CARE CENTRE A

The demographic data for the multiple surveys of children attending day care centre A was statistically analyzed to determine whether any risk factors for CMV infection could be identified between CMV excretors and children not excreting the virus (Table 14). As with the previous studies reported in the literature on CMV excretion prevalence in children attending day care centres, no risk factors could be identified for CMV infection within this population when the demographic data for the children attending the surveyed Halifax day care centre.

TABLE 13 Statistical analysis of the demographic attributes to determine possible risk factors associated with CMV excretion in all children surveyed for presence of CMV in urine.

Each child in the survey has been used as one data point.

DEMOGRAPHIC ATTRIBUTES	TOTAL NUMBER	NUMBER POSITIVE (%)	STATISTICAL SIGNIFICANCE
MALE/FEMALE	58/63	7/12 (12/19%)	ns (1)
AGE GROUP IN YEARS			
< 1 1/2	8	1 (13%)	ns (2)
1 1/2 < 2 1/2	39	5 (13%)	
2 1/2 < 3 1/2	30	7 (23%)	
3 1/2 < 6	41	6 (15%)	
NUMBER OF SIBLINGS			
0	72	11 (15%)	ns (2)
1	44	7 (16%)	
2	5	1 (20%)	
MOTHER'S AGE IN YEARS			ns (2)
20 - 24	11	0 (0%)	
25 - 29	59	7 (12%)	
30 - 34	36	8 (22%)	
35+	18	3 (17%)	
FATHER'S AGE IN YEARS			ns (2)
20 - 24	5	0 (0%)	
25 - 29	27	3 (11%)	
30 - 34	31	5 (16%)	
35+	36	9 (25%)	
MOTHER'S EDUCATION			ns (2)
HIGH SCHOOL	17	2 (12%)	
POST HIGH SCHOOL	77	15 (19%)	
GRADUATE SCHOOL	18	1 (6%)	
FATHER'S EDUCATION			ns (2)
HIGH SCHOOL	16	2 (13%)	
POST HIGH SCHOOL	53	10 (19%)	
GRADUATE SCHOOL	29	5 (17%)	

ss - STATISTICALLY SIGNIFICANT

ns - STATISTICALLY NOT SIGNIFICANT

(1) - CHI-SQUARE TEST (DETERMINES WHETHER THE VARIABLES ARE INDEPENDENT)

(2) - MANN-WHITNEY TEST (DETERMINES WHETHER DIFFERENCES EXIST BETWEEN TWO POPULATIONS WITH RANKED ATTRIBUTES)

TABLE 13 Statistical analysis of the demographic attributes to determine possible risk factors associated with CMV excretion in all children surveyed for presence of CMV in urine (continued).

MOTHER'S OCCUPATION			ns (2)
UNEMPLOYED	3	1 (33%)	
NONPROFESSIONAL	22	4 (18%)	
PROFESSIONAL	47	9 (19%)	
STUDENT	25	3 (12%)	
FATHER'S OCCUPATION			ns (2)
UNEMPLOYED	3	0 (0%)	
NONPROFESSIONAL	23	6 (26%)	
PROFESSIONAL	38	8 (21%)	
STUDENT	29	2 (7%)	

TABLE 14 Statistical analysis for the risk factors associated with CMV excretion in children from day care centre A. In some attributes parental responses may have not been obtained.

DEMOGRAPHIC ATTRIBUTES	TOTAL NUMBER	NUMBER POSITIVE	STATISTICAL SIGNIFICANCE
MALE/FEMALE	39/33	5/8 (13/24%)	ns (1)
AGE GROUP IN YEARS			ns (2)
< 1 1/2	8	1 (13%)	
1 1/2 < 2 1/2	27	4 (15%)	
2 1/2 < 3 1/2	15	5 (33%)	
3 1/2 < 6	19	3 (16%)	
NUMBER OF SIBLINGS			ns (2)
0	46	7 (15%)	
1	22	5 (23%)	
2	4	1 (25%)	
MOTHER'S AGE IN YEARS			ns (2)
20 - 24	11	0 (0%)	
25 - 29	27	4 (15%)	
30 - 34	22	7 (32%)	
35+	8	2 (25%)	
FATHER'S AGE IN YEARS			ns (2)
20 - 24	5	0 (0%)	
25 - 29	18	3 (17%)	
30 - 34	18	3 (17%)	
35+	19	6 (32%)	
MOTHER'S EDUCATION			ns (2)
HIGH SCHOOL	12	2 (17%)	
POST HIGH SCHOOL	50	11 (22%)	
GRADUATE SCHOOL	5	0 (0%)	
FATHER'S EDUCATION			ns (2)
HIGH SCHOOL	10	2 (20%)	
POST HIGH SCHOOL	38	7 (18%)	
GRADUATE SCHOOL	12	3 (25%)	

ss

- STATISTICALLY SIGNIFICANT

ns

- STATISTICALLY NOT SIGNIFICANT

(1)

- CHI-SQUARE TEST (DETERMINES WHETHER TWO VARIABLES ARE INDEPENDENT)

(2)

- MANN-WHITNEY TEST (DETERMINES WHETHER DIFFERENCES EXIST BETWEEN TWO POPULATIONS WITH A RANKED ATTRIBUTE)

TABLE 14 Statistical analysis for the risk factors
associated with CMV excretion in children from
day care centre A (continued).

MOTHER'S OCCUPATION			ns (2)
UNEMPLOYED	3	1 (33%)	
NONPROFESSIONAL	19	2 (11%)	
PROFESSIONAL	25	7 (28%)	
STUDENT	17	2 (12%)	
FATHER'S OCCUPATION			ns (2)
UNEMPLOYED	3	0 (0%)	
NONPROFESSIONAL	10	4 (40%)	
PROFESSIONAL	20	6 (30%)	
STUDENT	22	1 (5%)	

D. MOLECULAR EPIDEMIOLOGY OF THE CYTOMEGALOVIRUS ISOLATES

The epidemiological relatedness of the day care centre CMV isolates was ascertained by use of restriction endonuclease analysis. The electrophoretic fragment patterns, produced by various restriction enzyme digestions of the CMV DNA, were compared to determine whether the CMV isolates represented epidemiologically related CMV isolates of identical strains or unique nonepidemiologically related CMV strains. Epidemiologically unrelated CMV isolates are identified by heterogeneous (unique) electrophoretic fragment patterns produced by restriction enzymes. As previously described, restriction endonuclease analysis has been shown to be a useful epidemiological tool in determining whether CMV transmission has occurred in a population and in the comprehension of the modes of CMV infection.

CMV strains are at least 80% homologous; the electrophoretic fragment patterns produced by *EcoRI* or *BamHI* restriction endonuclease digestion exhibit extensive similarities in the electrophoretic fragments patterns (Kilpatrick et al 1976). Migration of DNA fragments to identical positions in the patterns does not necessarily represent base sequence identity. The position of a fragment depends on the molecular weight, not basepair identity. It is possible that fragments migrating to

identical positions within a pattern represent nonhomologous DNA sequences. Extensive matching of the electrophoretic patterns, however, would indicate significant similarity of the virus genomes (Kilpatrick et al 1976). Specific fragments or groups of matching fragments among different CMV isolates were proposed to represent constant regions of the CMV genome, possibly regions responsible for the basic CMV characteristics. The heterogeneity in DNA fragment patterns of the CMV isolates was determined to be the loss or gain of a small number of restriction sites but not to be the rearrangement of the gene(s) order as previously proposed by Huang et al (1980B) (Colimon et al 1985).

In this study the determination of the epidemiological relatedness of CMV isolates was originally based on Adler's (1985A) definition. The presence of one to two additional fragments in the electrophoretic fragment pattern (two or less band difference) was considered to be a minor variation and the isolates under comparison to belong to the same CMV strain. A major variation was defined as a difference of more than two fragments in the electrophoretic patterns, representing epidemiologically unrelated strains of CMV. Minor shifts in band location has been shown to occur with HSV electrophoretic fragment patterns, these are not thought to represent the major variations required to define epidemiologically distinct strains (Lonsdale et al 1980). A modification to Adler's (1985A) definition was

required during this study. Adler's analyses usually consisted of one enzyme (*EcoRI*) when this definition was proposed. This study utilized a number of restriction endonucleases for the assessment the CMV isolates. In a few cases varying results for the epidemiological relatedness of a CMV isolate were encountered when the individual restriction analysis results were compared. An overall assessment definition, therefore, was required.

For each restriction endonuclease digestion (independent on the restriction enzyme utilized) the resultant fragment band patterns were classified into four categories based on the differences found between the CMV isolates (modified from Adler 1985A). These categories were as follows; (1) no difference - no fragment pattern differences: identical CMV isolates, (2) few differences - one to two band differences: minor variation (possibly identical CMV isolates), (3) intermediate differences - three or four band differences: different CMV strains, and (4) - many differences - more than four band differences: different CMV strains. The intermediate variation category, defined to be a difference of three or four fragments, was important in those isolates that were found to have varying restriction analysis results requiring further restriction endonuclease analysis information prior to deciding the epidemiological identity of the CMV isolates. When the epidemiologically relatedness of such an isolate was

assessed, the result was determined by the majority of the individual restriction analyses results.

Superinfection, that is, the concurrent presence of two or more strains of CMV infecting an individual and therefore excretion of two or more CMV strains in a single sample, was excluded by comparing the restriction endonuclease patterns of the DNA from the first isolate to those of the remaining isolates (Adler 1988, Murph and Bale 1988). If the patterns remained identical to the initial pattern during future isolations, then the child was assumed to be excreting the same virus during the course of the survey and was said to not have been newly infected by a different CMV strain. However, if the individual was originally concurrently infected with two strains and no fragment pattern changes occurred during future isolations, then the individual would still be superinfected with at least two CMV strains and could only be identified as not having been infected with a different CMV strain during the study.

The number of restriction enzymes utilized in the comparisons of the day care centre isolates was determined by a review of the literature involving restriction endonuclease digestion of CMV samples to determine the number and identity of the enzymes utilized (Table 3). In the analysis of reported CMV surveys, only the isolates from related people or the same person have identical

electrophoretic fragment patterns with three or more restriction endonucleases (Huang et al 1980A & B). There was considerably more difficulty to establish genetic homology or identity between CMV isolates than to prove strain heterogeneity. Most researchers used three restriction enzymes to establish the epidemiological identity of the CMV isolates. The most common restriction enzymes used were *Bam*HI, *Hind*III and *Eco*RI. Peckham et al (1986) noted that, although three or four enzymes are routinely used to determine epidemiological relatedness of CMV isolates, analysis by one enzyme was usually sufficient to identify epidemiologically unrelated CMV isolates. The CMV genome consists of approximately 230 kb. The restriction sites for the commonly used enzymes to analyze the CMV genome consist of 6 nucleotide pairs, so only approximately 0.1% of the viral genome is specifically selected by each restriction endonuclease during digestion of the CMV genome (Peckham et al 1986).

*Eco*RI has been determined to be an unsatisfactory enzyme for epidemiological analysis of CMV isolates (Huang et al 1980A & B). *Eco*RI easily exhibits "star activity", the indiscriminate cleavage of DNA when restriction conditions vary slightly (Huang et al 1980 A & B, Maniatis et al 1982). Minor variations in the electrophoretic fragment patterns were found when DNA was analyzed by *Eco*RI as opposed to *Hind*III. The differences in the restriction

endonuclease patterns were not equivalent to the differences found with heterogeneous strains of CMV. When *EcoRI* was used as the restriction endonuclease, these strains were found to have variations in their electrophoretic fragment patterns. Due to the variations found in the patterns produced by *EcoRI*, this enzyme was not used for the analysis of the CMV isolates, instead *KpnI* was chosen to be the third enzyme, in addition to *BamHI* and *HindIII*. CMV isolates found to be the same by these three enzymes were further examined with two other restriction endonucleases, *SalI* and *XhoI*, to provide further evidence that the isolates were epidemiologically related.

For most of the restriction enzymes (*BamHI*, *HindIII*, *SalI* and *XhoI*) a DNA/enzyme ratio of 3.3 Units (U) enzyme per 1 ug DNA (50 U/15 ug) was used. The exception to this ratio was the restriction enzyme *KpnI*, for which a ratio of 1.6 U of enzyme per 1 ug DNA was employed. Varying DNA/enzyme ratios were tested. Although no partial digestions of DNA were found, if the DNA concentration was too high the restriction enzyme digestion of the DNA did not occur. The optimum electrophoretic fragment patterns were found when approximately 15 ug of DNA was digested by 50 U of enzyme.

The disadvantages in the restriction endonuclease analysis technique for determining epidemiological

relationships among CMV isolates include CMV cell culture techniques, the length of time to propagate enough virus to perform the analysis, and obtaining purified DNA. The CMV isolates, when first propagated from the urine samples obtained from the day care centre children, were kept in cell culture over extended time periods in order to allow the virus CPE to spread such that at least 50% of the cell monolayer showed evidence of infection. Often time periods of months elapsed during this incubation. The cultures were maintained by weekly medium changes. The possibility of contamination constantly existed. If the culture had become contaminated the virus isolate would be lost. Fortunately this did not occur. Only one virus specimen was lost during propagation: DC 15 (second isolate); however, contamination was not responsible. The virus isolate did not withstand transfer to new cell monolayers during the propagation process. From the initial urine specimen to the restriction endonuclease analysis of the DNA isolated from that specimen, a time period of six months to one year may have elapsed.

The CMV DNA sample purity was another problem encountered with the restriction endonuclease analysis of the day care centre CMV isolates. Host DNA and RNA and degraded nucleic acid were found to contaminate the CMV DNA sample. Multiple ethanol precipitations were not performed due to the small amounts of DNA samples, and the fact that

degraded nucleic acid was thought to be responsible for the heavy smear found in electrophoretic fragment patterns.

1. TRITIATED THYMIDINE RESTRICTION ANALYSIS

Visualization of the DNA electrophoretic fragment patterns following restriction endonuclease digestion of CMV DNA may be achieved by a variety of techniques including labelling viral DNA with radioisotopes, staining the DNA fragments and probe hybridization. During the initial stages of this study, the tritiated thymidine technique to label DNA was selected as the method of DNA band imaging. McFarlane and James (1984) devised a methodology to incorporate tritiated thymidine into HSV DNA and subsequently modified the methodology for analysis of CMV isolates (Embil et al 1985B, McFarlane and Koment 1986). The protocol for tritiated thymidine labelling of CMV DNA involved the addition of the label to the cell culture medium bathing a CMV infected HNFF monolayer. After a period of time the cells were harvested to isolate the viral DNA. The amount of viral DNA sample used in the restriction endonuclease analysis and the length of exposure time for the X-ray were both based on the amount of label incorporated into the CMV DNA sample. The CMV DNA label incorporation was determined by scintillation count. With HSV DNA, the optimum sample contains 15,000 to 20,000 counts

per minute with subsequent exposure of the film to the dried gel for approximately three days at -70°C (McFarlane and James 1984). HSV halts cellular DNA synthesis so that the label is incorporated only into the viral DNA. CMV does not halt cellular DNA synthesis, therefore, the label is incorporated into both host and viral DNA. Small amounts of host mitochondrial DNA, are found to contaminate the CMV DNA samples isolated by the HIRT method (Eizuru et al 1984). Therefore, the estimates of the incorporated label into the CMV DNA were not accurate. Unfortunately when the methodology was applied to the day care centre CMV isolates, obscure electrophoretic fragment patterns were obtained. The problems encountered with the restriction endonuclease digestion of tritiated thymidine labelled CMV DNA included light or no fluorographic image and low number of radioactive counts incorporated into the viral DNA sample.

No correlation seemed to exist between the amount of label incorporated in the sample DNA and the intensity of the resultant fluorographic electrophoretic fragment pattern. CMV DNA samples with low levels of label incorporated sometimes gave extremely good electrophoretic fragment patterns; whereas, samples with label levels in the "normal" range often appeared to be too light to distinguish the patterns. The amount of the DNA sample calculated to give optimum patterns did not correlate with the fluorographic patterns produced. Low label incorporation

resulted, in some samples, in a clear visible electrophoretic fragment pattern but a higher level of incorporation produced a very faint pattern in other samples.

Due to the overall low level of label incorporation in the CMV DNA, concentration of the DNA samples by an ethanol precipitation (Sealey and Southern 1982) was utilized as an attempt to improve the electrophoretic fragment patterns. Increased counts of approximately 4X were obtained. The ethanol precipitation, unfortunately, produced smaller DNA samples, thus, limiting the number of DNA restriction endonuclease digestions that could be performed on the virus DNA sample. Although success in obtaining electrophoretic fragment patterns for the CMV isolates intermittently occurred, the quality of the electrophoretic fragment patterns would not allow the detailed analysis required to determine the epidemiological relatedness of the CMV isolates.

A possible explanation for the ineffectiveness of the tritiated thymidine DNA labelling technique may be found with the growth of the day care centre CMV isolates. These isolates were extremely slow in producing CPE within the HNFF monolayers. A possible low concentration of virus within the urine of the asymptomatic children may have been responsible for the delayed CMV production (not confirmed by

plaque assay). Even after passage of the virus to amplify the amount of virus, the production of CPE within the monolayers still remained quite slow. This may partially account for the poor performance of the tritiated thymidine DNA labelling technique. During the methodology when the viral CPE involved approximately 75% of the cell monolayer, the label is placed onto the cells within a fresh change of cell culture medium which is then left for approximately two weeks (or until the cell monolayer appears to require a medium change). The medium was not replaced after the label was placed on the monolayer. If the production of viral CPE is quite rapid then a good uptake of the label into the newly synthesized viral DNA can be expected. However, if the viral production of CPE is quite slow then a lower level of label uptake within the same time period would be expected, resultant in lower levels of label incorporation into the CMV DNA. The methodology did not involve multiple label inoculations, although this may have been a possible solution to overcome the problems encountered with this technique.

When the effectiveness of this methodology was assessed, the inconsistency in producing usable results determined that the tritiated thymidine methodology (without modification) was not appropriate for analysis of the day care centre CMV isolates. At this point a different method to visualize the DNA fragments was employed: the ethidium

bromide staining methodology of DNA electrophoretic band patterns. The decision to use the ethidium bromide method was based on a number of factors. Simplicity of use and less time required for analysis (days versus months) were the two foremost reasons.

2. ETHIDIUM BROMIDE RESTRICTION ANALYSIS

The ethidium bromide DNA staining technique of visualizing the electrophoretic fragment patterns in agarose gels is a less sensitive technique for viral DNA detection than the tritiated DNA labelled technique. The methodology is based on the fact that ethidium bromide complexes with DNA and this complex is fluorescent when exposed to an ultraviolet light source (Sealey and Southern 1982). A photograph is used to record the electrophoretic fragment patterns.

Compensation for the lower sensitivity in detecting the DNA bands consisted of a greater quantity of virus was harvested by the use of multiple Falcon flasks of CMV infected monolayers per CMV isolate and the use of sample concentration by ethanol precipitation. Ethidium bromide staining of DNA fragments requires a large amount of DNA (2 ng in a 0.5 cm band) (Maniatis et al 1982), however, trailing and smearing in the gel tract will occur if the band concentration contains more than 200 ng of DNA. An

Initial DNA amount of 2 ug is required during restriction endonuclease digestion of the CMV genome due to the production of a large number of bands (Eizuru et al 1984). Although the spectrophotometric analysis determined that the DNA samples were relatively pure, in many cases a fluorescent background smear was visible. This background smear was thought to have been caused by degraded DNA and/or RNA. The smear interfered with the visibility of the electrophoretic fragment patterns. When the negative of the gel was printed, the background smear, in some cases, obscured the electrophoretic fragment pattern even though the bands were visible on the negative.

The clarity of the individual electrophoretic fragment patterns of restriction endonuclease digested CMV genomes varied within the sample. Within a CMV DNA sample, in some cases, highly visible electrophoretic fragment patterns were detected and in other cases very faint patterns were seen. The reason why the variation occurred is not known. However, this clarity variation may be due to possible pipetting inaccuracies and inconsistent DNA distribution in the sample causing altered DNA concentrations. Not all fragment patterns obtained were able to be used in the comparisons of the CMV isolates from the day care centre children.

When the DNA of an adenovirus isolate was digested by

restriction endonucleases in the same manner as the CMV isolates, very bright electrophoretic fragment bands were seen. Staining of the DNA bands by ethidium bromide binding in agarose gels has been determined to be a function of the length of the DNA and its concentration. The intensity of the band is a function of the molecular weight of the DNA and its molarity (Somogyi et al 1986). The concentration of DNA in the adenovirus sample was in the same range as the CMV samples. The molecular weight of CMV is approximately six times the molecular weight of adenovirus (150×10^6 as opposed to 25×10^6) (Joklik et al 1980); however, the restriction endonucleases only cuts the adenovirus DNA into less than 10 bands (usually 4 to 6 bands) as opposed to approximately 20 to 30 fragments as with the CMV isolates. As a result the bands exhibited by the CMV isolates are much fainter than the bands found with the adenovirus isolate.

Two DNA standards were included in most of the restriction endonuclease analysis gels: bacteriophage lambda and CMV DNA. These DNA standards had a number of functions. One such function was to establish that the conditions for restriction endonuclease cleavage of the DNA were correct during the enzyme digestion. The second function was to ensure that the ethidium bromide staining procedure worked. The DNA bands produced by the restriction endonuclease digestion of the DNA standards also served as reference points to compare the electrophoretic fragment patterns for

the CMV isolates contained on different gels. When the negatives of the CMV electrophoretic fragment patterns were printed, the distance between a bacteriophage lambda reference band and the well origin was standardized for each gel, thus, aligning the CMV isolates' fragment patterns for the common bands.

The electrophoretic fragments from the bacteriophage lambda DNA standard were not used as molecular weight markers to identify the CMV electrophoretic fragments. Researchers employing restriction endonuclease analysis to study the epidemiology of CMV assess the overall fragment pattern for the presence of differences within the pattern (Grillner and Blomberg 1984, Garret and Warren 1985, Peckham et al 1986). The DNA fragments found to vary are not usually identified as to which part of the CMV genome they belong.

Numerous attempts by many different photographic methods were made in order to obtain the clearest possible photographs of the electrophoretic fragment patterns. Photographic techniques were used to enhance the faint bands at the bottom of the gel. In fact, the negatives were printed by two professional technical photographers in hopes of improving the photograph quality, but even these attempts did not further increase the resolution of the electrophoretic fragment patterns. When the composite

photographs were produced, a further reduction in electrophoretic fragment pattern clarity was encountered. The contrast within the photograph increased; however, pattern resolution decreased as the white background was enhanced. Bands that were slightly visible over the white background smear became clouded by the increased contrast of the smear. Unfortunately in some cases the clarity of the patterns exhibited by the photographs did not reflect that of the photograph negatives. As previously stated, the analysis for epidemiological relatedness of the day care centre CMV isolates was based on the photographic negatives not the prints.

a. REPRODUCIBILITY OF ELECTROPHORETIC FRAGMENT PATTERNS

One of the underlying assumptions of the use of restriction endonuclease analysis to determine the epidemiological relatedness of CMV isolates is that a CMV isolate's electrophoretic fragment pattern produced by a specific enzyme remains consistent when the assay is repeated. Under a number of conditions the electrophoretic fragment patterns produced by the restriction endonuclease digestion of the CMV DNA were reproducible. When identical restriction endonuclease digested CMV DNA samples were analyzed in the same agarose gel, in different gels and, also, using different isolates of the same CMV strain (during different surveys from an individual child),

identical electrophoretic fragment patterns were displayed.

Electrophoretic fragment patterns were also found to be identical when different volumes of the DNA from a CMV isolate were individually digested by the restriction endonuclease *XhoI*. An ethidium bromide stained agarose gel following the electrophoresis of *XhoI* digested CMV DNA (DC 82) isolated from a day care centre child is presented in Figure 11. The electrophoretic fragment patterns for each concentration of DNA were identical in fragment pattern when digested with 50 U of *XhoI*. Pattern intensity varied considerably with the different DNA concentrations of sample DNA, although this photograph print does not accurately exhibit the variation. In order to exhibit the identical electrophoretic fragment patterns in each lane, the photograph was printed to have almost identical pattern intensities in lanes A, B, and C.

Prior to performing the comparative gels in which all the day care centre CMV isolates were analyzed to determine the epidemiological relatedness of the isolates, the restriction enzyme *XhoI* was used to determine the optimum volume of CMV viral DNA sample, and hence the concentration, to be used in the digestion reaction. This volume along with the DNA concentration, determined by spectrophotometry, was used to approximate the volume of sample required for the remaining restriction enzymes. A limited amount of

FIGURE 11: Agarose gel electrophoresis of DNA fragments of DC 82 CMV DNA digested with *Xho*I.

A photograph of an ethidium bromide stained agarose gel following electrophoresis of the DNA fragments produced by the digestion of DC 82 CMV DNA with the enzyme *Xho*I. Varying volumes of the same DNA isolate were individually digested by the restriction endonuclease *Xho*I for the different wells of an individual agarose gel to determine optimum DNA concentrations. The total volume of the reaction mixture was 102 μ l.

Lane A	-	DC 82	87 μ l	(39 μ g DNA)
Lane B	-	DC 82	60 μ l	(26 μ g DNA)
Lane C	-	DC 82	40 μ l	(18 μ g DNA)
Lane D	-	DC 82	20 μ l	(9 μ g DNA)
Lane E	-	DC 82	10 μ l	(4 μ g DNA)
Lane F	-	Bacteriophage lambda	2 μ l	(4 ng DNA)



sample was available for restriction endonuclease analysis so each specimen could not be analyzed with every restriction enzyme by this method. An example of this methodology is presented in Figure 11. Varying amounts of the DC 82 DNA isolate were analyzed, from 10 ul of sample containing 4 ug of DNA to 87 ul of sample containing 39 ug of DNA, by an individual digestion with 50 U of the restriction endonuclease *XhoI*. The resultant electrophoretic fragment patterns for each digestion of DC 82 DNA were found to be identical. Even with the larger amounts of DNA digested, no extra bands were found, indicating that partial digestion of the DNA by the restriction endonuclease had not occurred. Because the suggested DNA concentration of 10 ug per restriction endonuclease digestion (Maniatis et al 1982) did not always produce visibly clear ethidium bromide stained electrophoretic fragment patterns, all CMV isolates were analyzed by this method. The combination of the DNA concentration information obtained by spectrophotometry and the results obtained from the *XhoI* restriction endonuclease digestion of the varying amounts of CMV DNA was used to determine the optimum concentration of CMV DNA for the comparative restriction endonuclease analysis. For DC 82 this would be 30 ul of sample containing 15 ug DNA.

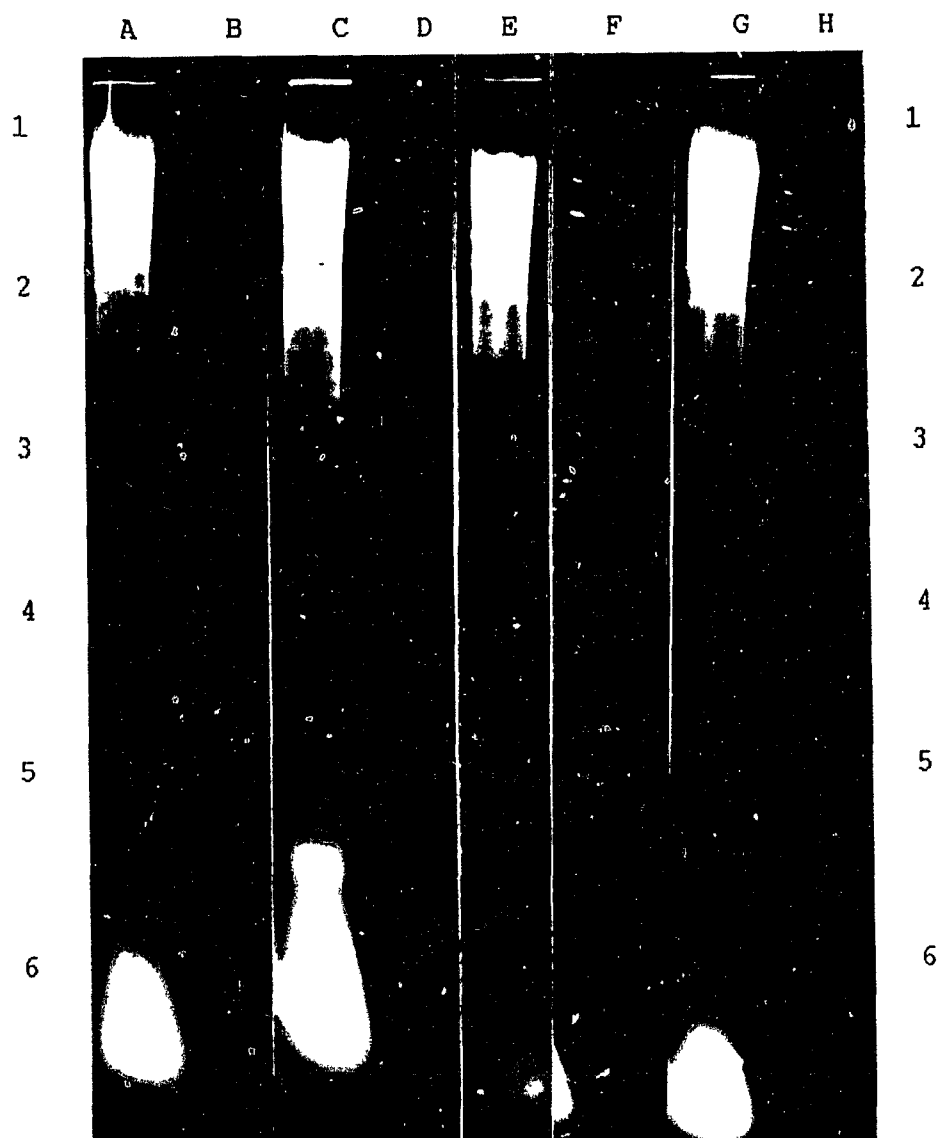
The electrophoretic fragment patterns, produced by restriction endonuclease digestion from the same CMV DNA

analyzed on separate occasions on different gels, were also identical (Figure 12). The overall length of the DNA band pattern from the well to the last visible band may change; however, the DNA fragment number and spatial proportion between the bands remained consistent. Separate restriction endonuclease analyses, of the DNA from the same preparation of isolate DC 100, on different agarose gels were performed on four separate occasions between February 1988 and May 1988. In each digestion, 30 ul containing 15 ug of DNA was digested by 25 U of the restriction enzyme KpnI. A bacteriophage lambda DNA standard (200 to 400 ng) was also included in each analysis. Changes in the electrophoretic fragment pattern length were seen with both bacteriophage lambda and DC 100 samples, however, the actual fragment patterns remained the same. Reference numbers have been placed on the sides on the photograph against which comparisons may be made. The well origins have been positioned in similar locations across the top of the photograph. The horizontal electrophoresis tank system employed during this study was not a submerged system, but rather a system in which the glass plate supporting the agarose gel is suspended between the two buffer reservoirs. The buffer is drawn from the reservoirs by wicks and a piece of plastic wrap keeps the agarose gel from drying out overnight. This system is sensitive to atmospheric conditions and, possibly, this resulted in the differing electrophoretic fragment pattern lengths.

FIGURE 12: Agarose gel electrophoresis of DNA fragments of DC 100 CMV digested with *Kpn*I.

A composite photograph of ethidium bromide stained DNA electrophoretic fragment patterns from agarose gels produced by the digestion of the DNA from the CMV isolate DC 100 obtained from day care centre A with the enzyme *Kpn*I. DC 100, as well as being an isolate from day care center A, was used as the CMV standard against which the electrophoretic patterns were compared. Different restriction endonuclease digestions were conducted during a five month time period, using the same isolate of DC 100 and bacteriophage lambda for each of the four gels, produced identical electrophoretic fragment patterns for each digestion. (Numbers have been placed on each side of the photograph to be used as points to refer to when comparing the electrophoretic band positions.)

Lane A - DC 100	Feb. 1988 (1)
Lane B - Bacteriophage lambda	Feb. 1988 (1)
Lane C - DC 100	Feb. 1988 (2)
Lane D - Bacteriophage lambda	Feb. 1988 (2)
Lane E - DC 100	Apr. 1988
Lane F - Bacteriophage lambda	Apr. 1988
Lane G - DC 100	May 1988
Lane H - Bacteriophage lambda	May 1988



When multiple CMV isolates obtained over an extended time period (three years) from an individual child were analyzed, the electrophoretic patterns of the DNA fragments from each CMV isolated were identical (Figure 24). This indicated that the child excreted the same strain of CMV over an extended (three year) time period and, also, that the DNA isolation and digestion procedures were reproducible. No extraneous DNA fragments were produced during the DNA extraction procedure. The photographs of the ethidium bromide stained electrophoretic fragment patterns produced by the digestion of CMV (DC 21) from three separate isolations with the restriction endonucleases *Bam*HI and *Kpn*I were identical in the electrophoretic fragment patterns for each enzyme. In both instances, two lanes (A & B, D & E representing the 1984 and 1985 survey isolates) were run on the same gel and the remaining lanes (C & F representing the 1987 isolate) were run on a separate gel. For each enzyme (*Bam*HI and *Kpn*I), all the electrophoretic fragment patterns were identical for the three isolates. All other restriction endonuclease digestions of multiple CMV isolates obtained (DC 01, 14, 15 & 37) were identical when analyzed by restriction endonuclease digestion to the electrophoretic fragment patterns of the original isolate (Figures 21, 22, 23, and 25).

Eizuru et al (1984) determined that DNA samples prepared by the Hirt method could be contaminated by host mitochondrial DNA; however, the contaminating DNA did not interfere with CMV strain identification. A single extra band representing host

mitochondrial DNA was found when CMV was digested by *EcoRI* (Eizuru et al 1984). In Figures 17a&b two intense bands seen in the *HindIII* electrophoretic fragment digestion patterns of the CMV isolates, positioned corresponding to the second Bacteriophage lambda DNA fragment and midway between the third and fourth Bacteriophage lambda DNA fragments, are thought to represent host mitochondrial DNA. The electrophoretic fragment patterns of the multiple CMV isolates (DC 01, 14, 15, 21 & 37) were identical to those of the electrophoretic patterns of the original isolate (Figures 21, 22, 23, 24 and 25), therefore confirming the fact that the contaminating host DNA did not interfere with CMV strain identification.

b. CYTOMEGALOVIRUS ISOLATES FROM DAY CARE CENTRE C, D AND E

The CMV isolates from children attending day care centres C, D and E were analyzed with three restriction endonucleases, *BamHI*, *HindIII*, and *KpnI* to determine whether the CMV isolates represented identical or unique CMV strains (Figures 13, 14 & 15). The results of the comparisons of the photographic negatives are presented in Table 15. All six children excreting CMV attending these day care centres were found to be infected by different CMV strains. Electrophoretic fragment patterns for the enzyme *KpnI* were unobtainable for CMV isolates DC 73 and DC 76, even after multiple attempts. Using the maximum volume of sample possible, the electrophoretic fragment

FIGURE 13: Agarose gel electrophoresis of DNA fragments of CMV isolates from day care centres C, D, and E digested with *Bam*HI.

A composite photograph of an ethidium bromide stained agarose gel following electrophoresis of the DNA fragments produced by the digestion of the DNA from the CMV isolates obtained from day care centres C, D and E with the enzyme *Bam*HI. The electrophoretic fragment patterns for DC 76 (Lane D) are quite faint in this photograph and cannot be distinguished. The Bacteriophage lambda standard was not printed.

Lane A - DC 88
Lane B - DC 82
Lane C - DC 81
Lane D - DC 76
Lane E - DC 73
Lane F - DC 54



FIGURE 14: Agarose gel electrophoresis of DNA fragments of CMV isolates from day care centre C, D, and E digested with *Hind*III.

A composite photograph of an ethidium bromide stained agarose gel following electrophoresis of the DNA fragments produced by the digestion of the DNA from the CMV isolates obtained from day care centres C, D and E with the enzyme *Hind*III. Bacteriophage lambda DNA, 4 ng, was the control against which the electrophoretic patterns were compared.

Lane A - DC 88
Lane B - DC 82
Lane C - DC 81
Lane D - DC 76
Lane E - DC 73
Lane F - DC 54
Lane G - Bacteriophage lambda

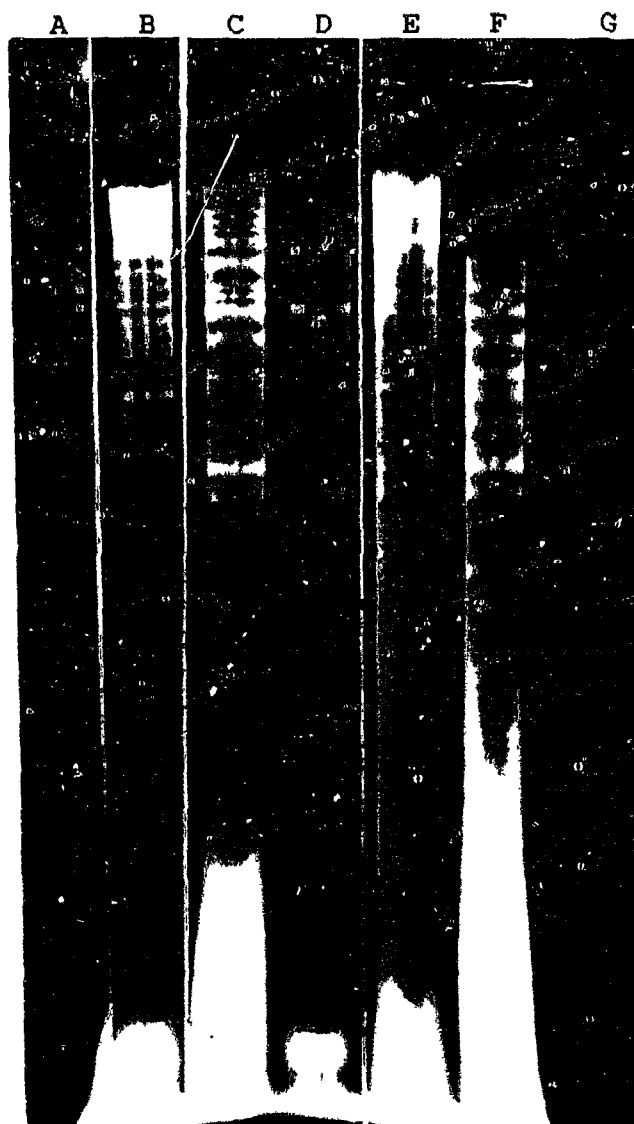


FIGURE 15: Agarose gel electrophoresis of DNA fragments of CMV isolates from day care centres C, D, and E digested with *Kpn*I.

A composite photograph of an ethidium bromide stained agarose gel following electrophoresis of the DNA fragments produced by the digestion of the DNA from the CMV isolates obtained from the day care centres C, D and E with the enzyme *Kpn*I. Bacteriophage lambda DNA, 4 ng, was used as a control against which the electrophoretic patterns were compared. Visible electrophoretic fragment patterns were not able to be obtained for the CMV isolates DC 73 and DC 76.

Lane A - DC 88
Lane B - DC 82
Lane C - DC 81
Lane D - DC 54
Lane E - Bacteriophage lambda

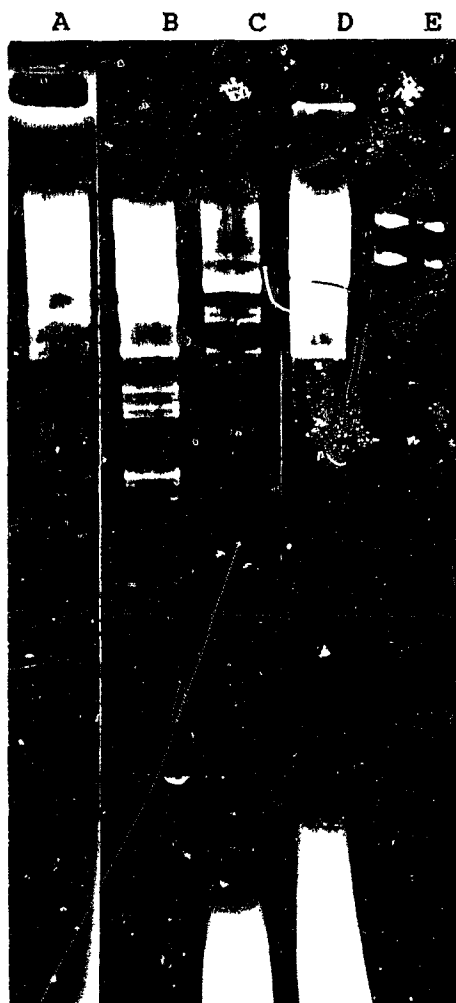


TABLE 15 Summary of the comparison of the CMV isolates from day care centres C, D and E by the photographic negatives of the electrophoretic fragment patterns.

		SPECIMEN NUMBERS				
		73	76	81	82	88
54	BAMHI	D	D	D	I	I
	HINDIII	D	D	D	D	D
	KPHI	?	?	?	D	D
73	BAMHI		D	I	I	D
	HINDIII		D	D	D	D
	KPHI		?	?	?	?
76	BAMHI			D	D	D
	HINDIII			D	D	D
	KPHI			?	?	?
81	BAMHI				D	D
	HINDIII				D	D
	KPHI				D	D
82	BAMHI					D
	HINDIII					D
	KPHI					D

ND- NO DIFFERENCES

F - 1 - 2 BANDS DIFFER

I - 3 - 4 BANDS DIFFER

D - MANY BANDS DIFFER

? - COULD NOT DETERMINE

patterns were consistently too faint to identify the isolates. The indistinct fragment patterns may have been due to overloading the enzyme with DNA or to a large number of bands produced by the digestion of the DNA. Although the *KpnI* restriction endonuclease analysis results were unavailable for the comparison of DC 73 and DC 76, the results for the restriction enzymes *BamHI* and *HindIII* digestions exhibited major differences. Based on the fragment pattern results produced by these restriction endonuclease digestions, all the CMV isolates were found to be unique (Table 15).

C. CYTOMEGALOVIRUS ISOLATES FROM DAY CARE CENTRE A

The CMV isolates from the children attending day care centre A were analyzed with up to four restriction endonucleases including *BamHI*, *HindIII*, *KpnI* (Figures 16 a & b, 17 a & b, 18 a & b) and *XhoI* (not shown). The results of the comparisons from the photographic negatives are presented in Table 16. In addition, isolates identical by *BamHI*, *HindIII* and *KpnI* were also analyzed with the enzyme *Sall*. Seven day care centre CMV DNA samples and two controls, bacteriophage lambda and CMV DC 100 filled the capacity of an agarose gel. As a result the day care centre CMV DNA fragment patterns had to be compared from multiple agarose gels. The fragment patterns from the different gels were analyzed by comparing individual DNA band locations to the comparable standard DNA bands.

FIGURE 16a: Agarose gel electrophoresis of DNA fragments of CMV isolates from day care centre A digested with *Bam*HI.

A composite photograph of ethidium bromide stained agarose gels following electrophoresis of the DNA fragments produced by the digestion of the DNA from the isolates obtained from day care center A with the enzyme *Bam*HI. The original day care centre isolates were used in this analysis. Bacteriophage lambda DNA, 4 ng, was a control and DC 100 (as well as being an isolate from day care centre A) was used as a CMV standard against which the electrophoretic patterns were compared; however, these two lanes were not printed in the composite. The individual gel photographs were aligned such that bacteriophage lambda's electrophoretic fragment patterns were equidistant from the well for each gel.

Lane A - DC 37
Lane B - DC 105
Lane C - DC 33
Lane D - DC 30
Lane E - DC 13
Lane F - DC 100
Lane G - DC 01
Lane H - DC 03
Lane I - DC 14
Lane J - DC 15
Lane K - DC 20
Lane L - DC 21
Lane M - DC 22

A B C D E F G H I J K L M

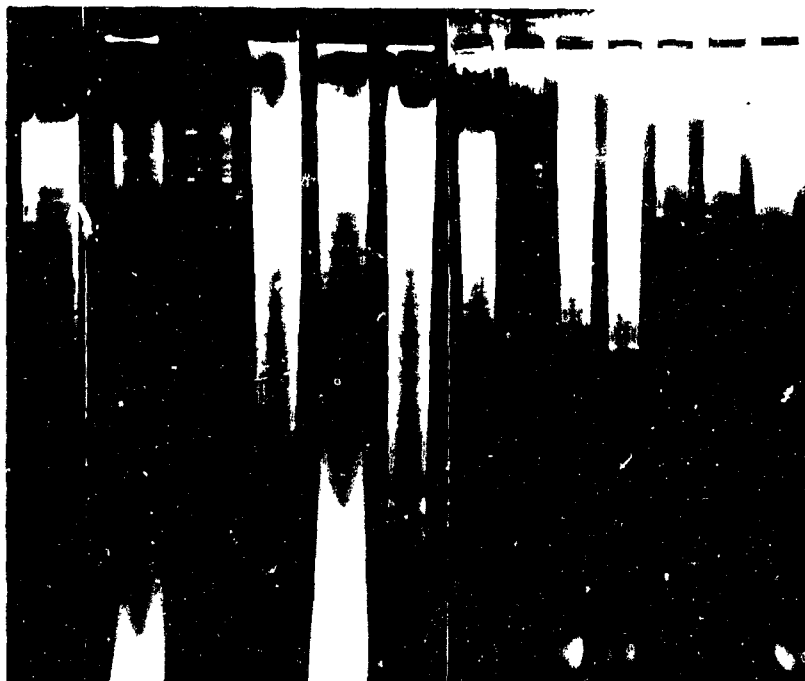


FIGURE 16b: Agarose gel electrophoresis of DNA fragments of CMV isolates from day care centre A digested with BamHI.

A composite photograph of ethidium bromide stained agarose gels following electrophoresis of the DNA fragments produced by the digestion of the DNA from the isolates obtained from day care centre A with the enzyme BamHI. The original isolates of each day care centre isolates were used in this analysis. Bacteriophage lambda DNA, 4 ng, was a control and DC 100 (as well as being an isolate from day care centre A) was used as a CMV standard against which the electrophoretic patterns were compared. The composite was not photographically altered to align the bacteriophage lambda fragments.

Lane A - DC 37
Lane B - DC 105
Lane C - DC 33
Lane D - DC 30
Lane E - DC 13
Lane F - DC 100 (CMV Standard)
Lane G - Bacteriophage lambda
Lane H - DC 22
Lane I - DC 21
Lane J - DC 20
Lane K - DC 15
Lane L - DC 14
Lane M - DC 03
Lane N - DC 01
Lane O - DC 100 (CMV Standard)
Lane P - Bacteriophage lambda

A B C D E F G H I J K L M N O P

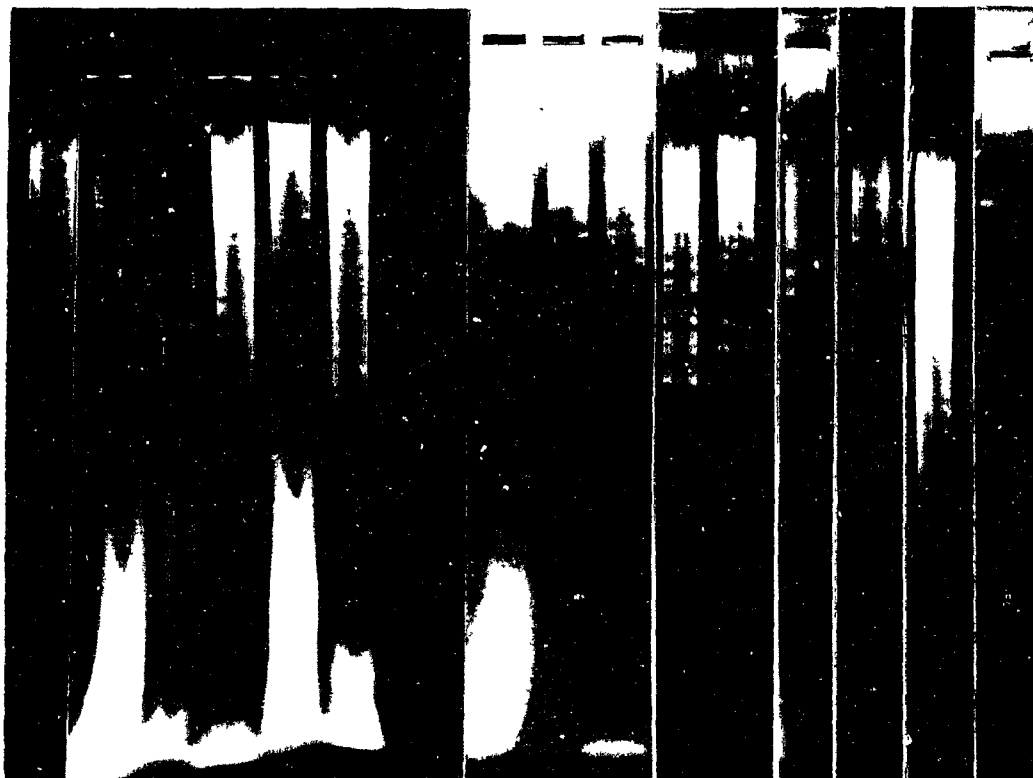


FIGURE 17a: Agarose gel electrophoresis of DNA fragments of CMV isolates from day care centre A digested with *Hind*III.

A composite photograph of ethidium bromide stained agarose gels following electrophoresis of the DNA fragments produced by the digestion of the DNA from the CMV isolates obtained from day care centre A with the enzyme *Hind*III. Bacteriophage lambda DNA, 4 ng, was a control and DC 100 (as well as being an isolate from day care centre A) was used as a CMV standard against which the electrophoretic patterns were compared. The individual gel photographs were aligned such that the bacteriophage lambda's electrophoretic fragment patterns were equidistant from the well for each gel.

Lane A - DC 105
Lane B - DC 37
Lane C - DC 33
Lane D - DC 30
Lane E - DC 13
Lane F - DC 100 (CMV Standard)
Lane G - empty
Lane H - Bacteriophage lambda
Lane I - DC 22
Lane J - DC 21
Lane K - DC 20
Lane L - DC 15
Lane M - DC 14
Lane N - DC 03
Lane O - DC 01

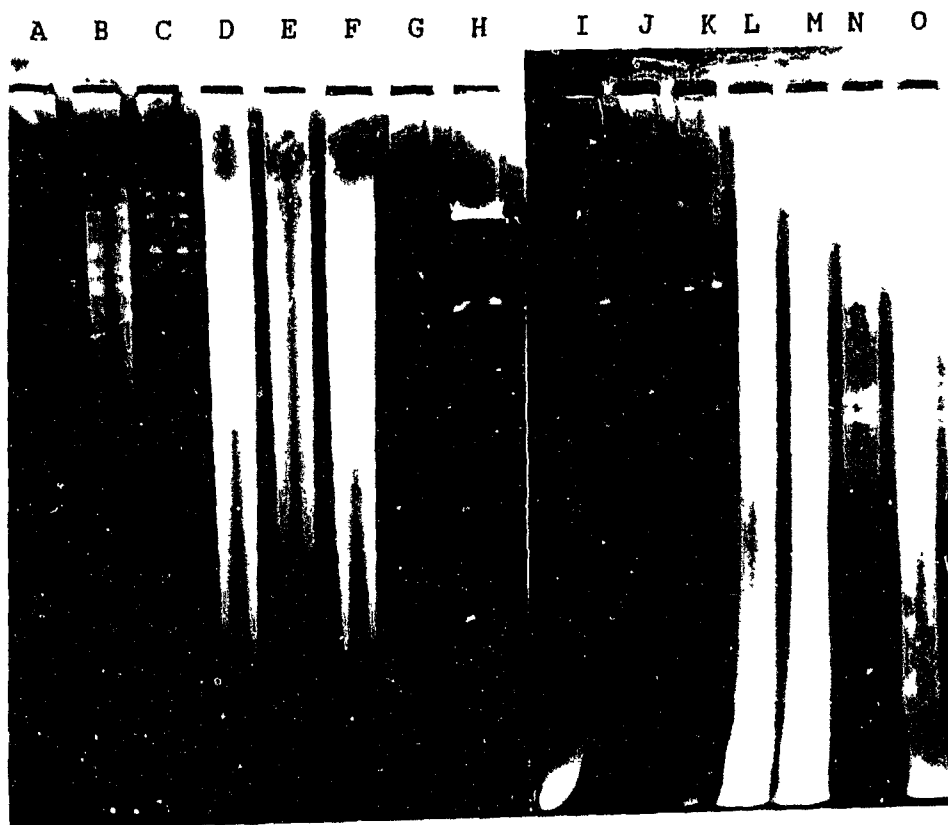


FIGURE 17b: Agarose gel electrophoresis of DNA fragments of CMV isolates from day care centre A digested with *Hind*III.

A composite photograph of ethidium bromide stained agarose gels following electrophoresis of the DNA fragments produced by the digestion of the DNA from the CMV isolates obtained from day care centre A with the enzyme *Hind*III. The original isolates of each day care centre isolate were used in this analysis. Bacteriophage lambda DNA, 4 ng, was a control and DC 100 (as well as being an isolate from day care centre A) was used as a CMV standard against which the electrophoretic patterns were compared. The composite was not photographically altered to align the fragments of bacteriophage lambda.

Lane A - DC 13
Lane B - DC 30
Lane C - DC 105
Lane D - DC 37
Lane E - DC 33
Lane F - DC 100 (CMV Standard)
Lane G - Lambda
Lane H - DC 22
Lane I - DC 21
Lane J - DC 20
Lane K - DC 15
Lane L - DC 14
Lane M - DC 03
Lane N - DC 01
Lane O - DC 100 (CMV Standard)
Lane P - Lambda

A B C D E F G H I J K L M N O P

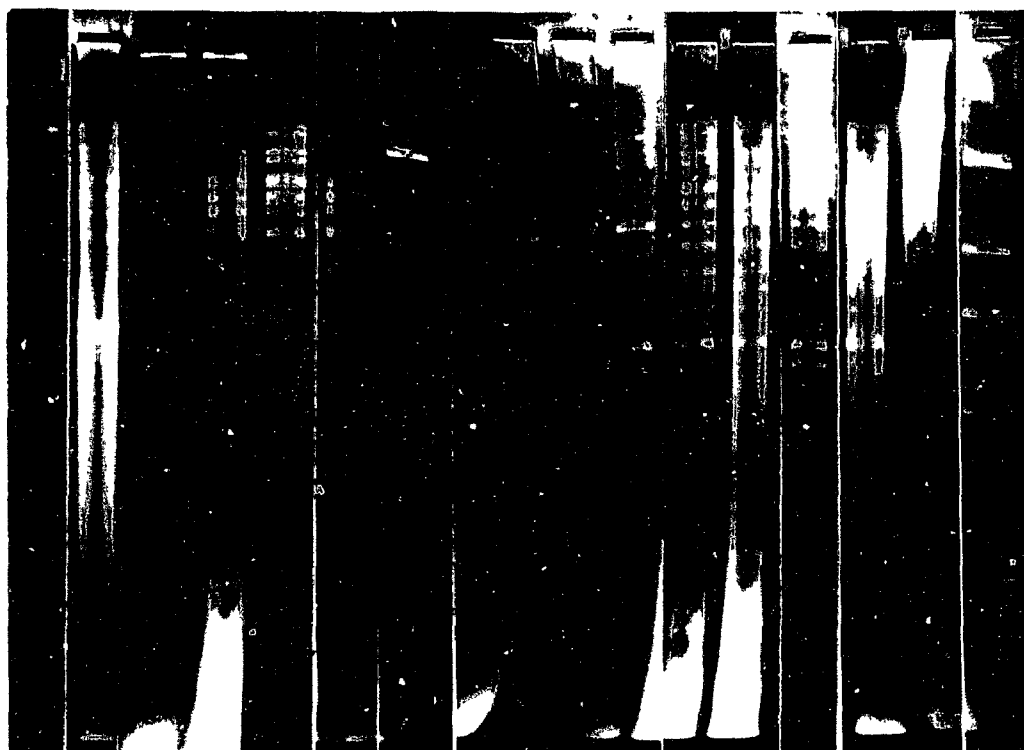


FIGURE 18a: Agarose gel electrophoresis of DNA fragments of CMV isolates from day care centre A digested with KpnI.

A composite photograph of ethidium bromide stained agarose gels following electrophoresis of the DNA fragments produced by the digestion of the DNA from the CMV isolates obtained from day care centre A with the enzyme KpnI. The original isolates of each day care centre isolate were used in this analysis. DC 100 (as well as being an isolate from day care centre A) was used as the CMV standard against which the electrophoretic patterns were compared. The individual gel photographs were aligned such that the electrophoretic fragment patterns of bacteriophage lambda were equidistant from the well of each gel.

Lane A - DC 22
Lane B - DC 21
Lane C - DC 20
Lane D - DC 15
Lane E - DC 14
Lane F - DC 03
Lane G - DC 01
Lane H - DC 105
Lane I - DC 37
Lane J - DC 33
Lane K - DC 30
Lane L - DC 13
Lane M - DC 100 (CMV Standard)
Lane N - Bacteriophage lambda



FIGURE 18b: Agarose gel electrophoresis of DNA fragments of CMV isolates from day care centre A digested with KpnI.

A composite photograph of ethidium bromide stained agarose gels following electrophoresis of the DNA fragments produced by the digestion of the DNA from the CMV isolates obtained from day care center A with the enzyme KpnI. The original isolates of the day care center isolates were used in this analysis. DC 100 (as well as being an isolate from day care center A) was used as the CMV standard against which the electrophoretic patterns were compared. The composite was not photographically altered to align the fragments of bacteriophage lambda. The electrophoretic fragment pattern for bacteriophage lambda was not printed.

Lane A - DC 37
Lane B - DC 33
Lane C - DC 22
Lane D - DC 21
Lane E - DC 20
Lane F - DC 13
Lane G - DC 30
Lane H - DC 15
Lane I - DC 14
Lane J - DC 03
Lane K - DC 01
Lane L - DC 100 (CMV Standard)



Table 16 Summary of a comparison of the restriction endonuclease digestion patterns following electrophoresis of the DNA for the isolates of day care centre A. The photographic negatives were used to make these determinations.

		SPECIMEN NUMBERS											
		03	13	14	15	20	21	22	30	33	37	100	105
01	BANHI	I	I	D	I	D	D	D	F	I	D	D	D
	HINDIII	D	F	D	F	D	D	?	D	D	?	D	?
	KPNI	D	D	D	D	D	D	D	D	D	D	D	?
03	BANHI		I	D	D	D	D	D	D	D	D	D	D
	HINDIII		D	D	D	?	?	?	D	D	D	D	?
	KPNI		D	D	D	D	D	D	D	D	D	D	?
13	BANHI			D	ND	D	D	D	D	D	D	D	D
	HINDIII			D	ND	?	?	?	D	D	D	D	?
	KPNI			D	ND	D	D	D	?	D	D	D	?
14	BANHI				D	D	ND	D	D	D	D	D	?*
	HINDIII				D	?	ND	?	D	D	F	D	?
	KPNI				D	D	ND	D	D	D	D	D	?
15	BANHI					D	D	D	D	D	D	D	D
	HINDIII					D	D	?	D	D	D	D	?
	KPNI					D	D	D	D	D	D	D	?
20	BANHI						D	D	D	D	D	D	D
	HINDIII						D	D	D	D	D	D	?
	KPNI						D	D	D	D	D	D	?
21	BANHI							D	D	D	D	D	?*
	HINDIII							D	D	D	D	D	?
	KPNI							D	D	D	D	D	?
22	BANHI								D	D	D	D	D
	HINDIII								?	?	?	?	?
	KPNI								D	D	D	D	?
30	BANHI									F*	D	D	D
	HINDIII									I	I	I	?
	KPNI									D	D	I	?
33	BANHI										D	D	D
	HINDIII										I	M	?
	KPNI										D	D	?
37	BANHI											D	D
	HINDIII											I	?
	KPNI											D	?
100	BANHI												D
	HINDIII												?
	KPNI												?

ND- NO DIFFERENCES

F - 1 - 2 BANDS DIFFER

I - 3 - 4 BANDS DIFFER

D - MANY BANDS DIFFER

? - COULD NOT DETERMINE

* - DIFFERENT BY XHO I

Two composite photographs (designated a & b) are presented for each enzyme analysis of the day care centre CMV isolates. The first photograph (a) displayed the photographically aligned fragment patterns. Unfortunately a clearly visible fragment pattern was not available for every CMV isolate. The second photograph (b) displayed the unaligned patterns. Unaligned fragment patterns in composite photographs were presented in Adler's 1985A and 1988 papers. The unaligned composite photographs consist of clearer patterns; therefore, they have been included for reference. By comparing the fragment patterns to the bacteriophage lambda reference fragments, evaluations may be made between CMV isolates.

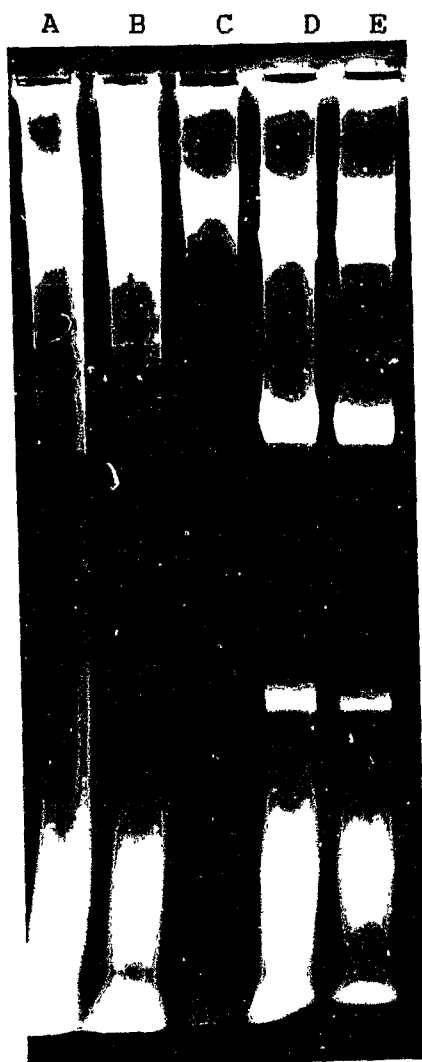
Of the 13 day care centre A children excreting CMV, seven children showed unique electrophoretic fragment patterns. Two children (DC 01 and DC 33) excreted viruses with similar, but not identical fragment patterns, to previously isolated viruses (DC 13 & 15 and DC 30). Two groups of two identical CMV isolates were identified (DC 15 & DC 13 and DC 14 & DC 21). Unfortunately, a few isolates' patterns were not obtained with a particular restriction enzyme. In those instances if major variations were found with any other restriction enzyme the isolate was considered to be epidemiologically unrelated (Table 16) (Peckham et al 1986).

A virus isolated from an infant was identified to be an adenovirus (Figure 19). The isolate was observed to have a

FIGURE 19: Agarose gel electrophoresis of DNA fragments of DC 30 CMV DNA and DC 23 DNA digested with XhoI.

A photograph of an ethidium bromide stained agarose gel following electrophoresis of the DNA fragments produced by the digestion of DC 30 DNA and DC 23 DNA with the enzyme XhoI.

Lane A	-	DC 30	87 ul	containing	19 ug	DNA
Lane B	-	DC 30	60 ul	containing	13 ug	DNA
Lane C	-	DC 30	40 ul	containing	9 ug	DNA
Lane D	-	DC 23	87 ul	containing	11 ug	DNA
Lane E	-	DC 23	60 ul	containing	8 ug	DNA



XhoI restriction pattern similar to that seen with adenovirus rather than the electrophoretic fragment pattern found with CMV isolates. Analysis with the various restriction enzymes *BamHI*, *EcoRI*, *HindIII*, *KpnI*, *SalI*, *XbaI*, *SmaI* and *XhoI*, identified the isolate as Adenovirus (Stewart 1984) (Figure 20).

Multiple isolates of CMV were obtained from five children (DC 01, DC 14, DC 15, DC 21 and DC 37) during the three surveys (Figures 21, 22, 23, 24 & 25). The CMV isolates from these children were analyzed by *BamHI*, *HindIII* and *KpnI* restriction enzyme digestions to determine if the electrophoretic fragment patterns had remained identical over the time period. If identical patterns occurred throughout the child's excretion of the virus, this would indicate that the child was excreting the same virus strain for an extended time period. For most of the photographs the CMV DNA samples were run on the same gel. In every case the electrophoretic fragment patterns remained identical. No minor variations of the virus genome were found. Two of the children (DC 15 and DC 21) were found to excrete the same CMV strain during all three of the surveys. Superinfection of these children with any new CMV strain may be excluded because the electrophoretic fragment patterns have remained constant (Adler 1988).

In one case (DC 15) all three urine samples collected produced CMV-CPE in the HNFF cell monolayers. The second survey CMV isolate produced the characteristic CPE in the HNFF

FIGURE 20: Agarose gel electrophoresis of DNA fragments of DC 23 DNA digested with eight enzymes.

A photograph of an ethidium bromide stained agarose gel following electrophoresis of the DNA fragments produced by the digestion of DC 23 DNA with eight enzymes. A sample volume of 40 μ l (5 μ g) was used for each digestion with DC 23. Bacteriophage lambda DNA, 4 ng, was used as a control against which the electrophoretic fragments were compared.

Lane A	-	DC 23	<i>Xho</i> I
Lane B	-	DC 23	<i>Sma</i> I
Lane C	-	DC 23	<i>Xba</i> I
Lane D	-	DC 23	<i>Sal</i> I
Lane E	-	DC 23	<i>Kpn</i> I
Lane F	-	DC 23	<i>Hind</i> III
Lane G	-	DC 23	<i>Eco</i> RI
Lane H	-	DC 23	<i>Bam</i> HI
Lane I	-	Bacteriophage lambda	<i>Bam</i> HI

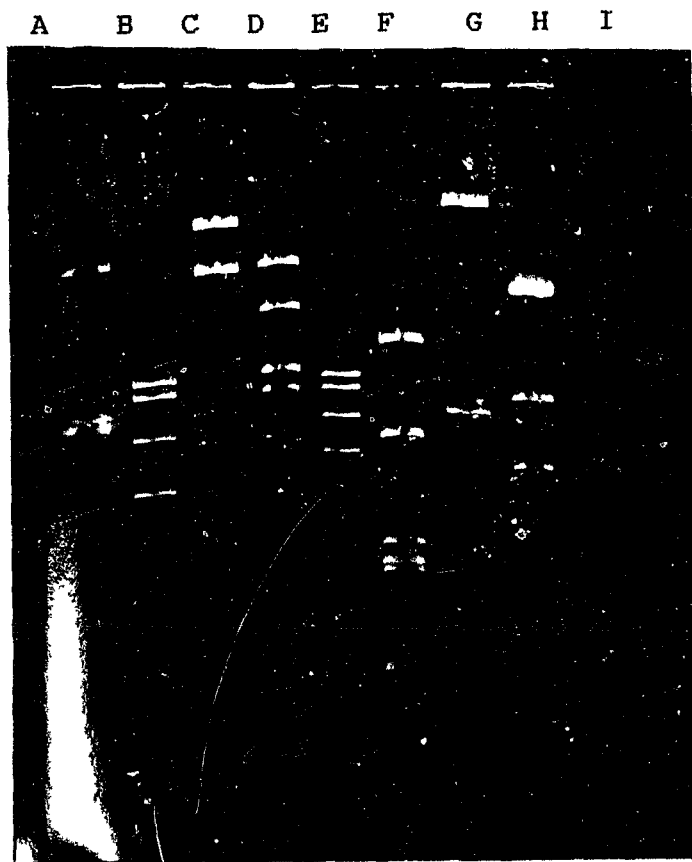


FIGURE 21: Agarose gel electrophoresis of DNA fragments from DC 01 CMV isolates obtained on different dates.

A photograph of an ethidium bromide stained agarose gels following electrophoresis of the DNA fragments produced by the digestion of the DNA from two isolates of DC 01 (day care centre A) obtained on different dates. The enzymes used were *Bam*HI, *Hind*III and *Kpn*I. Lane C and D were run on the same gel.

Lane A	- DC 01(Dec.10, 1984)	<i>Bam</i> HI
Lane B	- DC 01(Apr. 4, 1985)	<i>Bam</i> HI
Lane C	- DC 01(Dec.10, 1984)	<i>Hind</i> III
Lane D	- DC 01(Apr. 4, 1985)	<i>Hind</i> III
Lane E	- DC 01(Dec.10, 1984)	<i>Kpn</i> I
Lane F	- DC 01(Apr. 4, 1985)	<i>Kpn</i> I

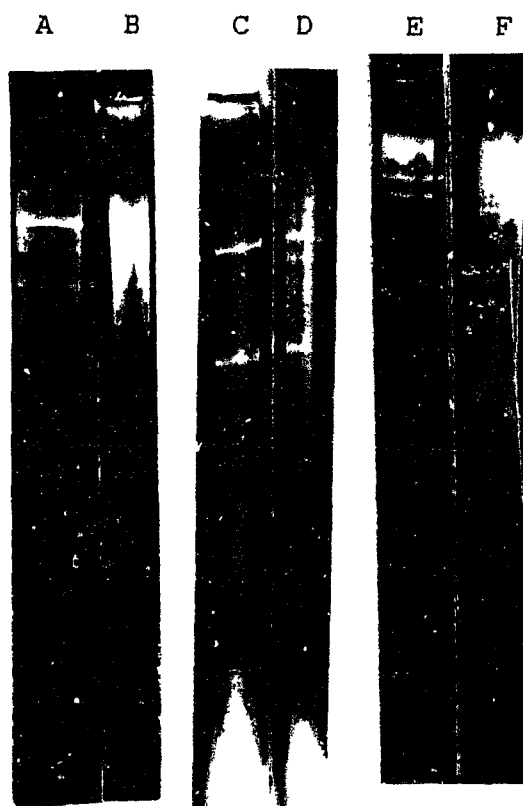


FIGURE 22: Agarose gel electrophoresis of DNA fragments from DC 14 CMV isolates obtained on different dates.

A photograph of ethidium bromide stained agarose gels following electrophoresis of the DNA fragments produced by the digestion of the DNA from two isolates of DC 14 (day care centre A) obtained on different dates. The enzymes used were *Bam*HI and *Kpn*I.

Lane A - DC 14(Dec.19, 1984) *Bam*HI

Lane B - DC 14(Jun.15, 1985) *Bam*HI

Lane E - DC 14(Dec.19, 1984) *Kpn*I

Lane F - DC 14(Jun.15, 1985) *Kpn*I



FIGURE 23: Agarose gel electrophoresis of DNA fragments from DC 15 CMV isolates obtained on different dates.

Photographs of ethidium bromide stained agarose gels following electrophoresis of the DNA fragments produced by the digestion of the DNA from two isolates of DC 15 (day care centre A) obtained on different dates. The enzymes used were *Bam*HI and *Kpn*I. A second photograph (A'B') of the *Bam*HI digests (unaligned) is presented for clarity. Lane C and D were run on the same gel.

Lane A -	DC 15(Dec.19, 1984)	<i>Bam</i> HI
Lane B -	DC 15(Mar 7, 1987)	<i>Bam</i> HI
Lane A'-	DC 15(Dec.19, 1984)	<i>Bam</i> HI
Lane B'-	DC 15(Mar 7, 1987)	<i>Bam</i> HI
Lane C -	DC 15(Dec.19, 1984)	<i>Kpn</i> I
Lane D -	DC 15(Mar. 7, 1987)	<i>Kpn</i> I



FIGURE 24: Agarose gel electrophoresis of DNA fragments from DC 21 CMV isolates obtained on different dates.

Photographs of ethidium bromide stained agarose gels following electrophoresis of the DNA fragments produced by the digestion of DNA from three isolates of DC 21 (day care centre A) obtained on different dates. The enzymes used were *Bam*HI and *Kpn*I. Lanes A & B and D & E were each run on the same gel.

Lane A - DC 21(Dec.19, 1984) *Bam*HI
Lane B - DC 21(Jun.15, 1985) *Bam*HI
Lane C - DC 21(Mar.19, 1987) *Bam*HI

Lane D - DC 21(Dec.19, 1984) *Kpn*I
Lane E - DC 21(Jun.15, 1985) *Kpn*I
Lane F - DC 21(Mar.19, 1987) *Kpn*I

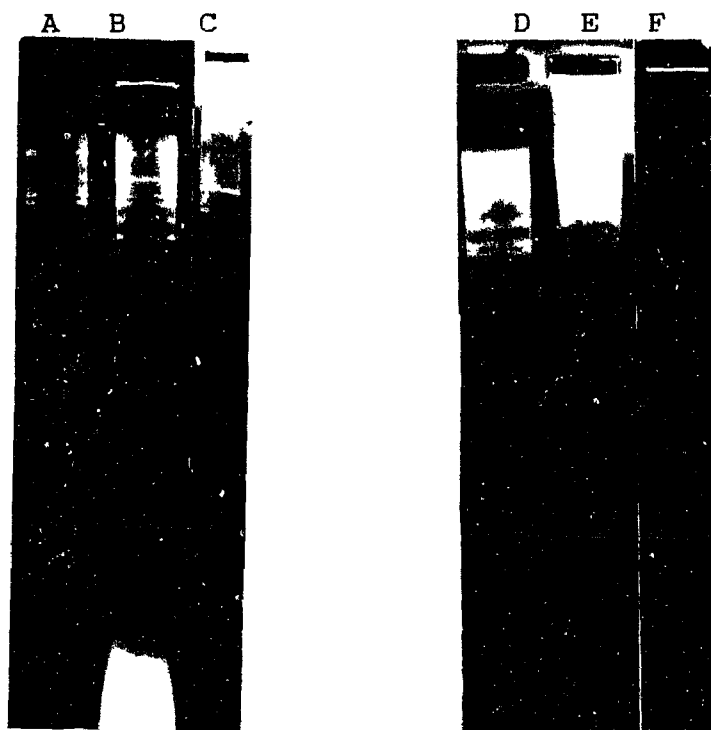


FIGURE 25: Agarose gel electrophoresis of DNA fragments from DC 37 CMV isolates obtained on different dates.

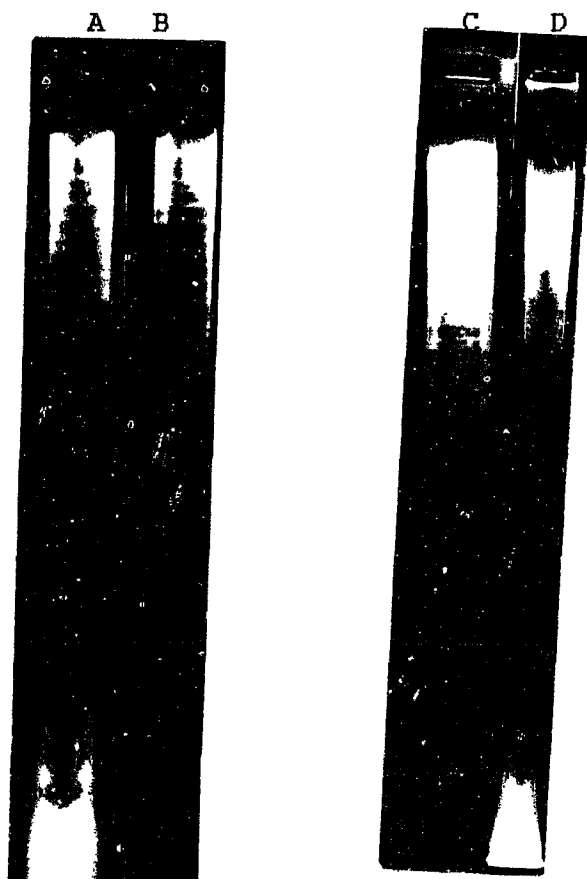
Photographs of ethidium bromide stained agarose gels following electrophoresis of the DNA fragments produced by the digestion of the DNA from two isolates of DC 37 (day care centre A) obtained on different dates. The enzymes used were *Bam*HI and *Kpn*I.

Lane A - DC 37(Jun.21, 1985) *Bam*HI

Lane B - DC 37(Mar. 5, 1987) *Bam*HI

Lane C - DC 37(Jun.21, 1985) *Kpn*I

Lane D - DC 37(Mar. 5, 1987) *Kpn*I



cell monolayer, however, the virus could not be recovered for restriction endonuclease analysis. The third survey isolate was able to be analyzed by restriction endonuclease analysis and found to be identical to the original isolate (Figure 23).

d. CYTOMEGALOVIRUS TRANSMISSION AMONG THE DAY CARE CENTRE CHILDREN

CMV transmission was found to occur among the children attending the Halifax day care centre A. Two groups of viruses were found, each group consisting of two identical virus isolates. In one case, viruses (DC 14 and DC 21) were concurrently isolated in the December 1984 survey from two members of the day care centre A toddler section (Figures 26 & 27). The viral DNA was analyzed by five restriction endonucleases, *Bam*HI, *Hind*III, *Kpn*I, *Sal*I and *Xho*I and, in each analysis, the two isolates were found to have identical electrophoretic fragment patterns indicative of epidemiologically related viruses. The progression of transmission could not be identified to determine which child infected the other due to the fact both children were concurrently found to be excreting virus.

In the second viral group, two identical CMV isolates were isolated during different surveys from children in two different day care centre divisions (DC 15, Toddler section and DC 13, Senior section). In the first survey, December

FIGURE 26: Agarose gel electrophoresis of DNA fragments from CMV isolates from two children attending day care centre A, DC 14 and DC 21.

Photographs of ethidium bromide stained agarose gels following electrophoresis of the DNA fragments produced by the digestion of the DNA from the CMV isolated from two different children attending day care centre A, DC 14 and DC 21. The enzymes used were *Bam*HI, *Hind*III, and *Kpn*I. Lanes C & D and E & F were each run on the same gel.

Lane A - DC 21 (Dec.20, 1984) *Bam*HI

Lane B - DC 14 (Dec.19, 1984) *Bam*HI

Lane C - DC 21 (Dec.20, 1984) *Hind*III

Lane D - DC 14 (Dec.19, 1984) *Hind*III

Lane E - DC 21 (Dec.20, 1984) *Kpn*I

Lane F - DC 14 (Dec.19, 1984) *Kpn*I



FIGURE 27: Agarose gel electrophoresis of DNA fragments from CMV isolates from two children attending day care centre A.

Photographs of ethidium bromide stained agarose gels following electrophoresis of the DNA fragments produced by the digestion of the DNA from the CMV isolates from two different children attending day care centre A, DC 14 and DC 21. The enzymes used were *Xho*I and *Sal*I. Lanes C & D were run on the same gel.

Lane A - DC 21 (Dec.20, 1984) *Xho*I
Lane B - DC 14 (Dec.19, 1984) *Xho*I

Lane C - DC 21 (Dec.20, 1984) *Sal*I
Lane D - DC 14 (Dec.19, 1984) *Sal*I



1984, a child in the Toddler section (DC 15) was found to be excreting CMV; whereas, the child attending the Senior section (DC 13) was found to be negative for CMV excretion. During the second survey, in June 1985, both children were identified to be excreting CMV. Restriction endonuclease analysis with the enzymes *Bam*HI, *Hind*III, *Kpn*I, *Sal*I and *Xho*I identified these two isolates as having identical electrophoretic fragment patterns with each enzyme, indicative of epidemiologically related viruses (Figures 28 & 29). During the initial 1984 survey, DC 15 was found to be excreting CMV and DC 13 was negative for CMV excretion, whereas during the second survey in 1985 both children were excreting CMV, therefore, it was thought that the child excreting DC 15 transmitted the virus to the child excreting DC 13.

Two children from day care centre A were found to have closely related CMV strains to other strains found within the day care centre population. One child (DC 01) had a similar virus to the DC 13 and DC 15 CMV strain. The restriction endonuclease electrophoretic fragment patterns showed only minor variations, that is less than three band differences, with *Hind*III. Intermediate variations, that is three to four band differences, were found with *Bam*HI and *Kpn*I (Figure 30, Table 16). The child (DC 01) is a sibling of the child excreting DC 13. Adler (1985A) described a major variation between CMV isolates (epidemiologically

FIGURE 28: Agarose gel electrophoresis of DNA fragments from CMV isolates from two children attending day care centre A, DC 13 and DC 15.

Photographs of ethidium bromide stained agarose gels following electrophoresis of the DNA fragments produced by the digestion of the DNA from the CMV isolated from two different children attending day care centre A, DC 13 and DC 15. The enzymes used were *Xho*I, *Sal*I and *Kpn*I. Lanes C & D and E & F were each run on the same gel.

Lane A - DC 13 (Jun.26, 1985) *Xho*I
Lane B - DC 15 (Dec.19, 1984) *Xho*I

Lane C - DC 13 (Jun.26, 1985) *Sal*I
Lane D - DC 15 (Dec.19, 1984) *Sal*I

Lane E - DC 13 (Jun.26, 1985) *Kpn*I
Lane F - DC 15 (Dec.19, 1984) *Kpn*I

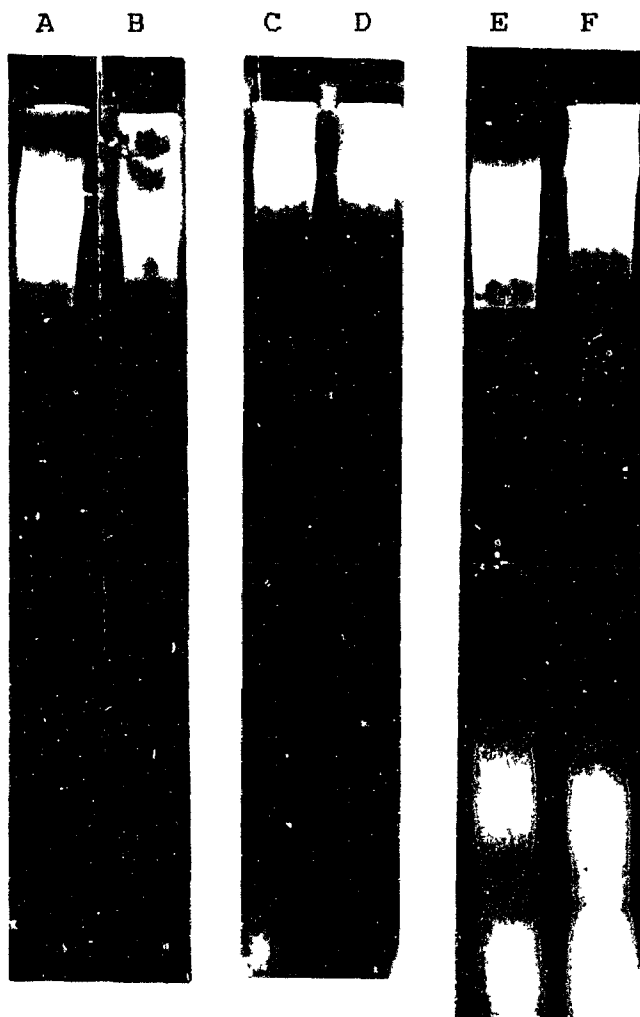


FIGURE 29: Agarose gel electrophoresis of DNA fragments from CMV isolates from two children attending day care centre A, DC 13 and DC 15.

Photographs of ethidium bromide stained agarose gels following electrophoresis of the DNA fragments produced by the digestion of the DNA from the CMV isolated from two different children attending day care centre A, DC 13 and DC 15. The enzymes used were *Bam*HI, and *Hind*III. Lane C & D were run on the same gel.

Lane A - DC 13 (Jun.26, 1985) *Bam*HI

Lane B - DC 15 (Dec.19, 1984) *Bam*HI

Lane C - DC 13 (Jun.26, 1985) *Hind*III

Lane D - DC 15 (Dec.19, 1984) *Hind*III



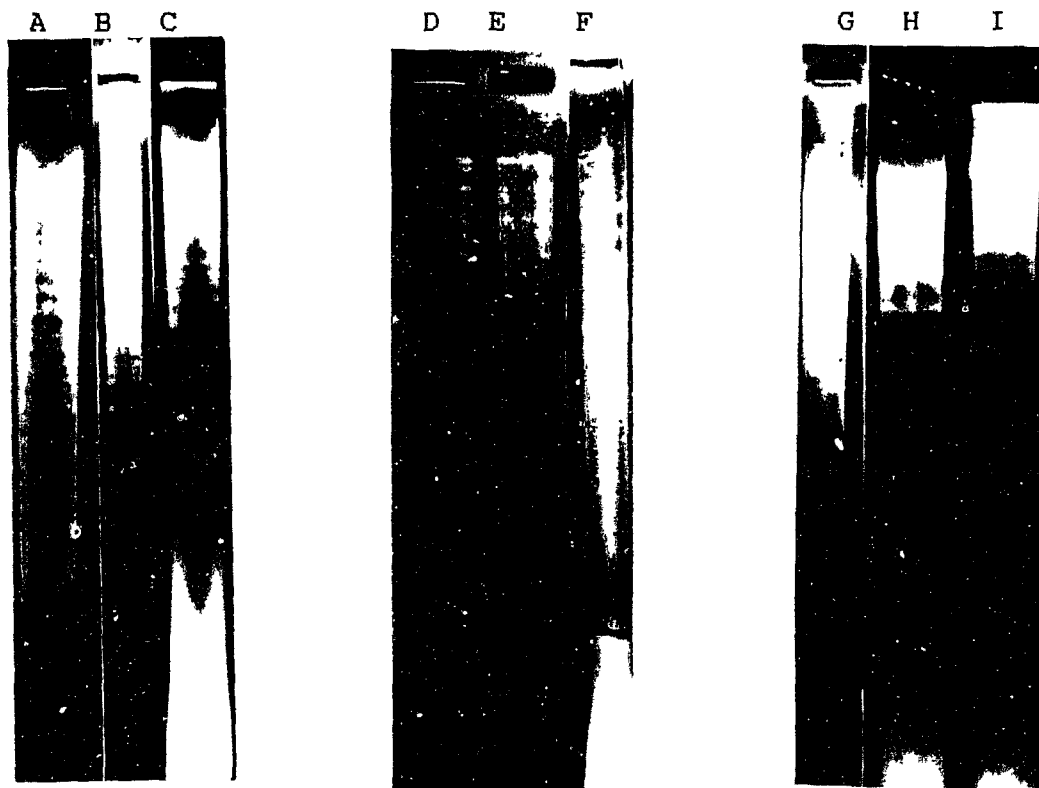
FIGURE 30: Agarose gel electrophoresis of DNA fragments from three children attending day care centre A, DC 01, DC 13, and DC 15.

Photographs of ethidium bromide stained agarose gels following electrophoresis of the DNA fragments produced by the digestion of the DNA from the CMV isolated from three different children attending day care centre A, DC 01, DC 13 and DC 15. The enzymes used were *Bam*HI, *Hind*III and *Kpn*I. Lanes D & E and H & I were each run on the same gel. Although these three viral isolates are very similar, DC 01 is not identical to DC 13 and DC 15.

Lane A - DC 01 (Dec.10, 1984) *Bam*HI
 Lane B - DC 15 (Dec.19, 1984) *Bam*HI
 Lane C - DC 13 (Jun.26, 1985) *Bam*HI

Lane D - DC 13 (Jun.26, 1985) *Hind*III
 Lane E - DC 15 (Dec.19, 1984) *Hind*III
 Lane F - DC 01 (Dec.10, 1984) *Hind*III

Lane G - DC 01 (Dec.10, 1984) *Kpn*I
 Lane H - DC 15 (Dec.19, 1984) *Kpn*I
 Lane I - DC 13 (Jun.26, 1985) *Kpn*I



unrelated isolates) as more than two band differences in any band position, this would classify the isolate as an epidemiologically unrelated CMV to DC 13 and DC 15. This CMV isolate is very difficult to classify.

Another child (DC 33) was found to have a closely related CMV strain to a strain previously found to be excreted by a child in the day care centre (DC 30) (Figure 31 & 32, Table 16). Both children attended the junior day care group together. Minor variations were found between the two isolates with the restriction enzyme *Bam*HI, intermediate differences were found by *Hind*III, and major variations were found by using the enzymes *Kpn*I and *Xho*I.

When the electrophoretic fragment patterns for the CMV isolates from day care centres C, D and E were compared with the electrophoretic fragment patterns for the CMV isolates from children attending day care centre A, the CMV isolates from day care centres C, D and E were all epidemiologically unrelated CMV strains.

FIGURE 31: Agarose gel electrophoresis of DNA fragments from CMV isolates from two children attending day care centre A, DC 30 and DC 33.

Photographs of ethidium bromide stained agarose gels following electrophoresis of the DNA fragments produced by the digestion of the DNA from the CMV isolated from two different children attending day care centre A, DC 30 and DC 33. The enzymes used were *Bam*HI and *Hind*III. Lane A & B and C & D were each run on the same gel. Although these three viral isolates are very similar, DC 30 is not identical to DC 33.

Lane A - DC 33 (Mar.10, 1987) *Bam*HI

Lane B - DC 30 (Apr.16, 1985) *Bam*HI

Lane C - DC 30 (Apr.16, 1985) *Hind*III

Lane D - DC 33 (Mar.10, 1987) *Hind*III



FIGURE 32: Agarose gel electrophoresis of DNA fragments from CMV isolates from two children attending day care centre A, DC 30 and DC 33.

Photographs of ethidium bromide stained agarose gels following electrophoresis of the DNA fragments produced by the digestion of the DNA from the CMV isolated from two different children attending day care centre A, DC 30 and DC 33. Lane A & B were run on the same gel. The enzymes used were *Kpn*I and *Xho*I. Although these three viral isolates are very similar, DC 30 is not identical to DC 33.

Lane A - DC 33 (Mar.10, 1987) *Kpn*I
Lane B - DC 30 (Apr.16, 1985) *Kpn*I

Lane C - DC 30 (Apr.16, 1985) *Xho*I
Lane D - DC 33 (Mar.10, 1987) *Xho*I



IV. DISCUSSION

Reseachers studying the epidemiology of CMV infections have shown interest in the presence of CMV within day care centre children populations. The day care centre environment allows children to congregate and usually represents the child's first encounter with large numbers of other children. Middle to upper socioeconomic family children represent a normal, low risk, immunocompetent population which, prior to the popularity of day care centres, had exhibited a low CMV prevalence. The increased CMV urine excretion prevalence within these children could possibly result in altering the existing epidemiology of CMV (Strangert et al 1976, Pass et al 1982 and 1984, Jones et al 1985, Nelson et al 1987). As previously described, CMV infection within United States day care centre populations is higher than expected for children of comparable age and socioeconomic status (Pass et al 1982, 1984). The increased CMV urine excretion prevalence within the children attending day care centres prompted concern for the possibility of the children infecting their parents, particularly, a pregnant seronegative mother with the potential consequence of a congenitally infected infant (Pass et al 1982). All previous studies for CMV prevalence in day care centre populations have been carried out in the United States,

British or Scandinavian day care centre populations, therefore Halifax, Nova Scotia, Canada, day care centre populations were examined to determine answers to the following questions.

A- The prevalence and incidence of urine excretion for CMV in a Halifax day care centre population. What were the individual frequencies for the day care centre populations observed and overall Halifax day care centre population CMV excretion? Do these Halifax day care centre populations differ from those found in the United States and Britain?

B- Could potential risk categories for Halifax day care populations (age, sex, socioeconomic status, siblings etc.) be identified?

C- Were strains of CMV able to be identified using restriction endonuclease digestion as an epidemiological tool? If CMV strains were found to predominate amongst the children attending a day care centre, did a strain have an increased infectivity shown by higher frequencies as compared to the other strains?

D- Did CMV transmission occur among the day care centre children?

The determination of CMV transmission within a given

population depended on a number of components, each necessary to proceed to the next stage of the study. A source of the virus had to exist within the population. The virus must be identifiable, for example visible by restriction endonuclease electrophoretic fragment patterns. The population must contain distinguishable, susceptible individuals. Newly infected individuals must be able to be detected and the virus must be able to be recovered. Finally, the virus must be identified as previously present within the given population.

This study on CMV transmission within the Halifax day care centre population fulfilled the requirements to determine whether transmission had occurred within the day care centre population. Initially, children excreting CMV were identified within a Halifax day care centre population. These children would be the potential source of virus to be transmitted to susceptible day care centre children. The electrophoretic fragment patterns produced by restriction endonuclease analysis of the virus DNA were used to determine the identity of the virus isolates. Susceptible children, that is children not excreting CMV, were detected within the day care centre population. A time period of at least six months was allowed to elapse prior to reexamining the population for evidence of virus transmission. Comparison of the electrophoretic fragment patterns produced from the new isolates of virus within the population against

the patterns from the existing isolates were analyzed to determine whether transmission of the virus among the children attending the day care centre had occurred.

Transmission of CMV among children at a day care centre was detected; however, it was found to be a rare event. Only two children were identified as being infected within the day care centre environment. The progression of virus transmission was able to be identified in one case. In the remaining case, both of the children concurrently excreted the same strain of virus; therefore, the transmission progression could not be determined.

Discussed below is the epidemiology of CMV within the surveyed Halifax day care centre populations.

A. CYTOMEGALOVIRUS URINE EXCRETION PREVALENCE AND INCIDENCE IN HALIFAX DAY CARE CENTRES

The CMV excretion prevalence found during the survey of Halifax day care centre children populations varied between 0% to 33% for the individual day care centres, with an overall prevalence of 13%, 95% confidence interval from 6% to 21%. This is lower than that reported in the literature. Several studies reported the CMV excretion prevalence in day care centre children populations to be higher than expected for that population (Pass et al 1982, 1984, Hutto and Pass

1984, Hutto et al 1985A, 1985B, Adler 1985A, Jones et al 1985, Murph et al 1986, Nelson et al 1987, Grillner et al 1988) and also higher than that of control children cared for at home (Pass et al 1984, Adler 1985A). This study did not consist of a case/control design; therefore, the question of whether the CMV urine excretion prevalence in Halifax day care centre children is elevated in comparison to children not attending day care was not directly assessed (Rationale discussed later).

An overall CMV excretion prevalence of 13% (95% confidence interval from 6% to 21%) was found for children attending the five Halifax day care centres. A total of 76 children were surveyed, ten children from four day care centres excreted CMV. It should be noted that this CMV excretion prevalence is a combined day care centre population total (Table 10). Most of the individual day care centre populations had CMV urine excretion prevalences ranging from 13% to 17%. Two of the day care centre populations, day care centre B and day care centre E varied from these prevalences exhibiting CMV urine excretion prevalences of 0% and 33% respectively. The low sample size is responsible for the large variation found in CMV prevalences.

A study objective was to estimate the CMV urine excretion prevalence in the Halifax day care centre

community. Approximately 77 children had to be sampled to determine the CMV excretion prevalence with 95% confidence coefficient within 8%. The CMV survey consisted of 75 children. Therefore, although the sample size of the day care centre children was small, enough children participated to give a statistically valid sample to determine the CMV excretion frequency. The power of the study to detect trends within the data was high (98).

The statistical analysis of the demographic descriptors for the five day care centres surveyed for CMV prevalence did not identify any descriptors to be significantly different with the exception of parental age. The children's age and the families' socioeconomic status (shown by previous studies of CMV prevalence in children to be important factors in CMV acquisition) were found to be similar in all the day care centre populations. Therefore, unless an unknown factor influenced the CMV transmission in these day care centres, it would be expected that the CMV urine excretion prevalence within the individual day care centre populations would be similar due to the similarities of the populations.

When the day care centres were categorized into registered and private day care centre groupings and the total CMV urine excretion prevalence determined for each group, the CMV prevalences were 11% (6 out of 57) for the

registered day care centres and 22% (4 out of 18) for the private day care centres. Although the private day care centre population's CMV prevalence appears to be considerably higher (22% versus 11%, respectively), chi-squared analysis determined that the prevalence difference between the two classifications of day care centres was not significant. The test power was 71. The populations attending the two different classifications of day care centres were also not found to be significantly different in the parental professions demographic descriptor. The socioeconomic status of the children's families should be quite similar. It would be expected that if the socioeconomic status was approximately the same for the two populations, the CMV prevalences would not be significantly different (unless another variable associated with CMV acquisition was altered). The results supported this statement, the CMV urine excretion prevalences were not significantly different between the populations attending the two classifications of day care centres.

The CMV urine excretion prevalence found in the Halifax day care centre populations was considerably lower than the reported prevalence for the United States, Britain and Swedish day care centre populations (as previously described in the Introduction). In the reported day care centre populations, the CMV urine excretion prevalence ranged from 57% to 20%, exceptions were 10% and 8% (Hutto et al 1985B).

Most of the United States day care centre children populations had a CMV urine excretion prevalence around 27%. In fact, the only survey to show low CMV urine excretion prevalences (10% and 8%) was Hutto et al's (1985B) survey involving two low socioeconomic day care centres. The sample sizes were not responsible for the lowered prevalences in these day care centres. A possible explanation for the increased CMV urine excretion prevalence in the United States day care centre children could be the size of the day care centres surveyed and, thus, the increased risk of a child attending the day care centre to have personal contact with a child excreting CMV (discussed later).

Not enough children (only seven) participated in all three surveys to make a valid estimation of the CMV urine excretion incidence in the day care centre population during the total survey period. As the project progressed it became evident that multiple surveys could not be executed in all the day care centres under observation. The time and number of samples were the factors that affected this decision. Day care centre A was selected for repeat sampling to determine if viral transmission had occurred among the day care centre children. It was also anticipated that CMV incidence of infection data would be obtained during the multiple surveys. This did not prove to be an accurate assumption. Day care centre populations are quite

mobile as seen by a large number of children leaving the day care centre. The children aged, progressing through the day care centre and finally leaving the day care centre to attend school. Parents either were transient, relocated their children to other day care centres or removed their children from day care to care for their children at home. As a result of this mobile population and the time required for the isolation of the virus, very few of the children included at the survey beginning remained throughout the duration. This fluctuation affected the data collected to determine the incidence of CMV within this population.

An estimate of the CMV urine excretion incidence within the day care centre population was made, however, for a six month interval. If the first two surveys of day care centre A are examined, 21 children participated in both surveys. During the first survey four children were identified to be excreting CMV. This left 17 children, the population at risk, susceptible to be infected by CMV. The second survey, six months later, identified four children to have newly acquired CMV infections. Incidence of infection is defined as the number of new cases of infection developed during a specified period of time as determined by viral excretion and/or the appearance of antibodies between two points of time. The day care centre A's CMV incidence would then be calculated as four CMV cases per six months. The CMV incidence rate would be the number of new cases (4) divided

by the population at risk (17) for the six month period or 0.24 cases per six months. This is based, however, on a very small sample size.

The parents of the day care centre children were not informed of the virus isolation results. This was partially due to the mobility of the population. By the time the virus isolation and restriction endonuclease confirmation of a positive CMV isolation had been concluded, possibly up to a time period of one year, many of the children had left the day care centre.

1. CYTOMEGALOVIRUS URINE EXCRETION PREVALENCE WITHIN THE DIFFERENT AGE GROUPINGS OF THE DAY CARE CENTRE CHILDREN

The CMV excretion prevalences for the different age groupings of children attending the Halifax day care centres were found to increase with age from ages 18 months to 42 months, then to decline during the ages 42 months to 60 months. Day care centre A was typical with this trend during the repeated samplings of the day care centre population (Figure 33). The children excreting CMV, consisting of six males and 13 females, were categorized into age groupings. The ages of the CMV-excreting children ranged from under 18 months to 5 years. One child excreting CMV was in the infant category, five of the children were between 18 months and 29 months, seven children were between

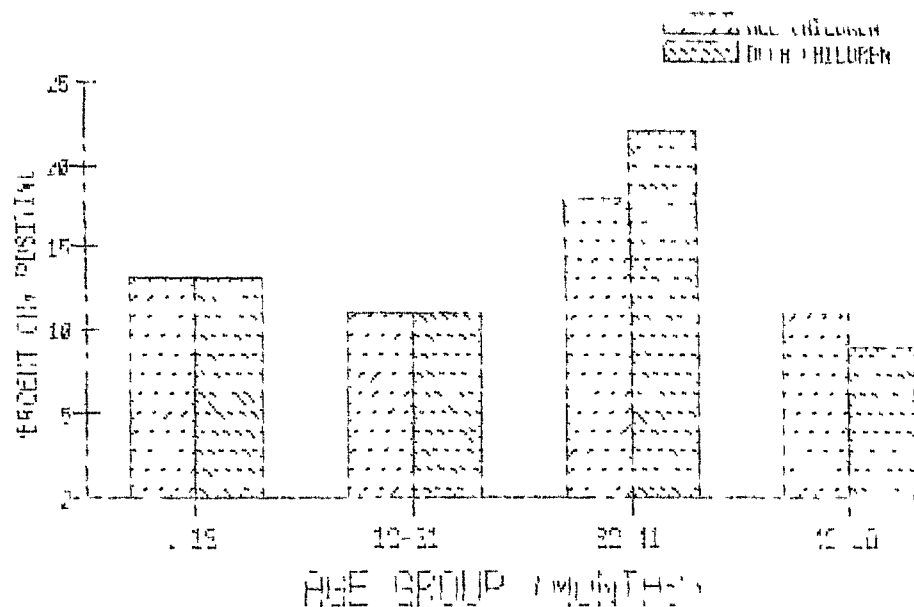


FIGURE 33 The CMV excretion prevalence in the different age groupings of Halifax day care centre children. The prevalence results were classified into two categories, all the children surveyed for presence of CMV and children attending day care centre A.

30 months and 41 months and the remaining six children were between 42 months and five years of age. The mean age of the children excreting CMV was calculated to be three years.

Children identified to be excreting CMV were more likely to be in the junior age group category, ages 32 months to 41 months. The increased CMV urine excretion prevalence at this age category is unlike the results found by Pass et al (1982, 1984) and Murph et al (1986). The highest CMV urine excretion prevalence found during those studies was exhibited by the toddler group children, aged 12 months to 24 months. Pass et al's (1982) classification of toddler children included children between the ages of 12 months to 36 months; whereas the toddler group category in this survey included children aged 18 to 31 months.

The age that the Halifax day care centre children first become infected with CMV could not be determined for the majority of the children examined. For this age to be identified, an initial CMV negative urine sample is required, followed by the identification of future CMV excretion. Three children, however, did meet this requirement. One child (DC 03) began to excrete CMV at twenty eight months of age while attending the toddler class at the day care centre. Another child (DC 33) attending the junior class began excreting CMV at three years of age. The remaining child (DC 13) aged fifty one months began to

excrete CMV while attending the senior section. When the initial CMV excretion began none of these children were between the ages of 12 months to 24 months, the age range which Pass et al (1982) proposed that most day care centre children acquired CMV infections. In fact, the three children represented nearly all the age groupings found in day care centre A, with only one child from the infant category included. By examining these three ages at which CMV excretion began and considering the fact that the highest CMV urine excretion prevalence was found in the day care centre children between the ages of 30 months to 41 months as opposed to 12 to 24 months, then the age at which children attending Halifax day care centres acquire CMV infections must be greater than the reported ages of day care centre children (Pass et al 1982, 1984, Murph et al 1986). The Halifax day care centres do not contain large numbers of children in the infant age category. A possible reason for the older age at which Halifax day care centre children become infected with CMV is that the children are older when they first attend day care centres and consequently are older when they first have close personal contact with large numbers of children and the possibility of encountering a CMV excreting child.

Pass et al (1982) proposed that the lower CMV prevalence in day care centre children aged 42 month to 60 months represents a cessation of CMV urine excretion by the

older children. The results of this study disagree with that proposal. Two children (DC 15 and DC 21) were found to continuously excrete CMV in their urine from the ages 24 months and 30 months, (toddler and junior day care centre groups, respectively) to ages 54 months and 60 months (senior day care centre group) after which the children left the day care centre. Only one child (DC 14) discontinued excreting CMV in its urine by the time attendance in the senior group occurred. This child was first identified as excreting CMV at age 30 months while attending the junior group at the day care centre and continued to excrete CMV at three years of age. When surveyed at age 54 months of age the child was no longer excreting virus. Because this survey did not follow the children after they left the day care centres, none of the children excreting CMV were followed past day care centre attendance to determine the age at which CMV excretion ceased.

A previous survey, approximately 20 years prior by Embil et al (1969A), determined the prevalence of CMV antibodies in blood samples in the general Halifax population (Figure 34). The survey population included children aged from newborn to 14 years of age. The antibody levels to CMV were found to decrease from birth to 2 years, representing the gradual loss of maternal antibodies to CMV, then increased to 16% from 2 years to 9 years. The highest antibody level increases representing increases in CMV

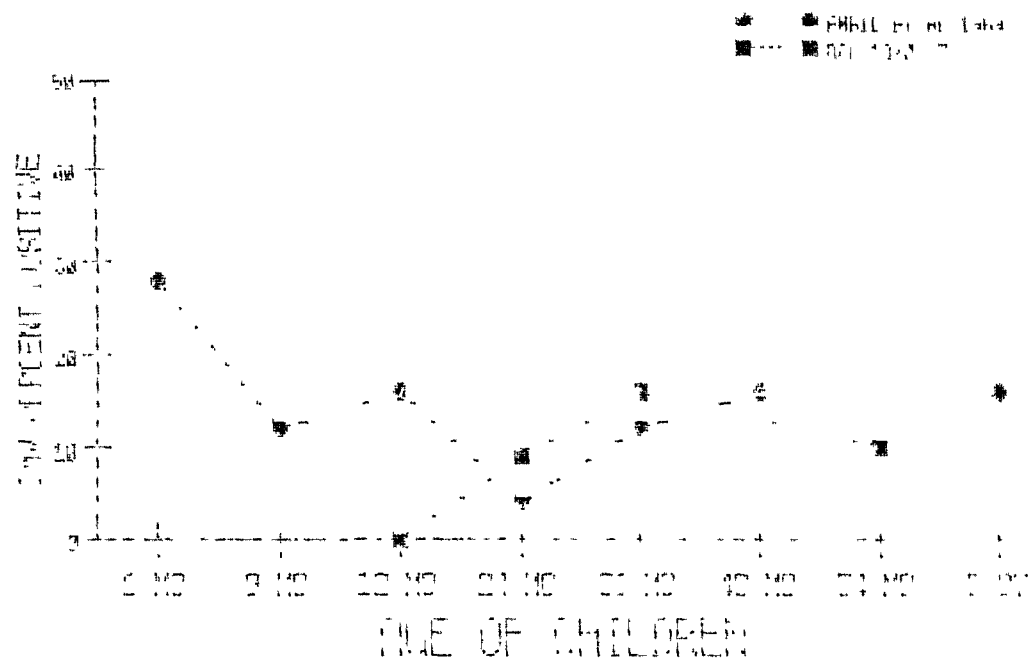


FIGURE 34 Comparison of Embil et al's 1969A serological survey of the general Halifax children population for presence of CMV antibodies to the results found during the urine excretion CMV survey of Halifax day care center children. The populations were not controlled for socioeconomic status, year, etc.

infections were found between the ages two to four years. This is the same age at which the day care centre children were found to have an increased CMV excretion prevalence. As previously stated CMV prevalence determined by CMV antibody levels and CMV urine excretion have been shown to be different (reviewed in the Introduction). The antibody levels would identify children with past, as well as, present infection; whereas, CMV excretion would only identify those individuals with a current infection (Pass et al 1982). Pass et al (1982) has suggested that a comparison could be made between the two types of prevalence estimates by increasing the value for the CMV urine excretion prevalence by approximately 10% to 30% in order to compare it to the serological CMV prevalence (Pass et al 1982). Even with this adjustment, the CMV excretion prevalence found in the day care centre children is not considerably higher than the percentage of children found to have antibodies to CMV reported during Embil et al's (1969A) survey approximately twenty years prior.

The comparison between the CMV prevalences obtained during this study of day care centre populations and the general Halifax population (Embil et al 1969A) did not adjust for socioeconomical status or other demographical attributes. The 1969 survey consisted of the general population found in Halifax-Dartmouth during the years 1967 and 1968. Day care centre use was not common in 1969 as in

current times. Based on the figures reported by Health and Welfare Canada (1987) at least an eightfold increase in the use of day care centres has occurred. Therefore, the children surveyed by Embil et al (1969A) would not have been likely to be exposed to the close daily contact with large numbers of other children that the day care centre environment provides. As previously stated, the CMV prevalence estimated by Embil et al's (1969A) survey represented the prevalence of CMV in various age groups of the general population. The Halifax day care centre children assessed in this study were mostly from middle socioeconomic families (parents with post high school education and professional occupations). The CMV prevalence for children of middle socioeconomic families should have had a lowered CMV prevalence than the general population surveyed for the 1969 study. When the data from the two surveys were compared, the day care centre children did not exhibit the large increase in CMV prevalence that Pass et al's 1982 and 1984 surveys found in Alabama day care centre children. Even within the United States day care centre populations, no other researcher (Adler 1985A & B, Jones et al 1985, Murph et al 1986) has found CMV prevalence rates in day care centre populations as high as those found by Pass et al (1982, 1984) (Table 2).

2. HALIFAX DAY CARE CENTER DESCRIPTIONS

a. SUBJECT PARTICIPATION

During the progression of this survey one aspect of epidemiological research became evident; the difficulty of acquiring subject participation. The survey was designed such that information letters and consent forms (Appendix 3) were distributed to the parents when they came to the day care centre to gather their children. It was anticipated that the parents would take the time to read the letter due to distribution of the letters by day care centre staff, consent to allow their child to participate in the project, complete the forms and return the forms to the day care centre. In order to generate a valid response, the letter was phrased in such a manner to explain to the parents that this survey would benefit future young children attending day care centres. The survey response varied from from 46% to 84%. These percentages are slightly lower than that reported in the literature; however the response is similar to published survey responses (Table 17). The realistic upper limit for a mail out questionnaire is 80%; however, the response is usually lower than that figure (Marks, 1982). It is anticipated that had a Public Health team of nurses and a physician performed general health assessments on the day care centre children, collecting the urine samples for CMV isolation during the assessment, the

TABLE 17 Reported day care centre parental responses to surveys.

A comparison of the reported responses to the surveys was undertaken to determine the parental participation to surveys within day care centres. In some of the reports the numbers of children participating in the surveys are not provided.

RESEARCHERS	NO. CHILDREN IN DAY CARE CENTRES	NO. CHILDREN PARTICIPATING	NO. CHILDREN PROVIDING SAMPLES
PASS ET AL 1982	75	70 (93%)	68 (97%)
HUTTO & PASS 1984	217		108 (90%)
HUTTO ET AL 1985A	188		155 (88%)
MURPH ET AL 1986	48 48		39 (81%) 41 (85%)
JONES ET AL 1985	156	106 (64%)	90 (85%)
THIS STUDY	191	106 (55%)	75 (71%)

parental response would have been considerably higher.

To identify whether differences existed in the number of responses between the parents of children attending the two classifications of day care centres, the day care centres were grouped into registered and private day care centres. The average responses were 62% and 78% respectively. Although the parents of children attending the private day care centre exhibited a slightly higher response frequency, the numbers of negative responses were also higher (Table 6). The parents of children attending the registered day care centre were more likely not to respond; however, of those that did return the forms, most submitted a positive response.

Unfortunately not all the children in each day care centre could be evaluated for presence of CMV. Only the children whose parents completed and returned a consent form expressing their permission to allow their child to participate in the survey were included. Therefore, a selection bias may have occurred. Children whose parents respond to surveys may be more concerned about children's health issues, more responsive to survey forms and more willing to allow their children to participate in such a survey. However, CMV transmission within day care centre populations has been shown to be influenced by close contact with the children during day care centre attendance (Pass et

al 1982, 1984), so that this bias should not have influenced the CMV urine excretion prevalence or transmission results.

As well as contending with the parent's cooperation, the issue of the children's compliance in providing a urine sample occurred. The number of children examined for urine CMV excretion was affected by children objecting to providing a urine sample. As previously described in the Materials and Methods section, if a child objected to providing the urine sample on three separate occasions, the child was omitted from the survey. During the first survey of day care centre A, some of the children attending the senior section refused to provide specimens. Another factor influencing the number of children willing to provide urine samples was participating children leaving the day care centre during the time period between the returning of the consent form and the collection of the urine samples.

b. DEMOGRAPHIC DESCRIPTOR ANALYSIS

The survey forms distributed to the parents of the children attending Halifax day care centres consisted of questions to determine the child's age, family size, parental age and the family's socioeconomic status based on the education and occupation of the child's parents. The socioeconomic status of the day care centre families was required in order to describe the day care centre's general

clientele. Controversial factors, such as race and breast feeding history, found not to be significant in previous studies (Pass et al 1982, 1984, Hutto et al 1985A) were omitted from the survey forms in hopes of generating a large parental response. Statistical analysis to analyze the demographic factors for all the day care centres used in the urine collection found no significant differences in the day care centre populations with the exception of parental age. The number of parents surveyed was large enough to give a statistically valid sample such that trends within the data would be detected.

When the numbers of children in attendance at each day care centre were examined it became apparent that the registered day care centres were larger in size than the private day care centres (Table 6). That factor may have introduced confounding, that is, the differences seen between the private and registered day care centres may be due to the larger number of children in attendance at the registered day care centres and not due to whether the day care centres were registered or private. When the day care centres were selected, a range of day care centre sizes were incorporated to prevent the size of the day care centre enrollment from influencing the results. Day care centre F had to be omitted from the survey as previously mentioned. The omission of this day care centre altered the size design of the study. Although confounding has the potential to

have occurred, evidence based on the CMV infection rates suggests that the results were not influenced.

3. RATIONALE FOR EXCLUDING STAFF AND HOME CARE CONTROLS

The unenthusiastic response from the day care centre staff regarding staff sampling was encountered at the origin of the survey. After the analyses of the CMV isolates obtained from the day care centre children were complete, no strain of CMV was found to be predominant. Therefore, the possibility of staff involvement in the transmission of CMV to the day care centre children should be quite low. In consideration of these two factors, it was decided not to survey the staff for presence of CMV excretion.

No attempt was made to determine the CMV prevalence in children not attending day care centres. A number of factors influenced this decision. The main reason was that proper control children are very difficult to obtain for a study such as this survey. In Pass et al's surveys (1982, 1984) healthy children attending a pediatrician's office in the same location of the day care centre were used as controls for the day care centre population. The basis for the use of the pediatric patients as the control population was the premise that the children attending the day care centre and the pediatrician's practice would be from approximately the same socioeconomic status, as the location

of the day care centre and the physician's practice were similar. This study of Halifax day care centre children found that children attending a day care centre were not necessarily from the same location as the day care centre. The determination of whether the pediatrician's practice described in Pass's study would include children from the same location as the day care centre would be difficult. The control children also included children exposed to day care centre environments for a limited amount of time. Children who would have the same increased contact with other children as the study population but for a lessor amount of time. Pass et al's (1982, 1984) estimate of the CMV prevalence for home care children should be inflated due to these children.

Adler (1985A) used hospitalized children as control children to determine the CMV urine excretion prevalence in children not exposed to a day care centre environment. This is an inappropriate control group. Hospitalized children include children who are immunocompromised and are in the high risk groups for acquisition of CMV infections. The CMV prevalences exhibited by these children would not reflect the CMV prevalence found in normal healthy children found in day care centres. Also, children who are long term patients may have decreased routine contact with other children and adults as found with a healthy child. The control population were also not socioeconomically matched with the

children found in the day care centre studied.

4. DO HALIFAX DAY CARE CENTRE POPULATIONS DIFFER FROM THOSE REPORTED IN THE LITERATURE?

Recommendations for prevention of CMV infections within day care centre populations are based on United States findings. The CMV excretion prevalence in the subset of Halifax day care centre populations was found to be lower than that reported for most day care centre populations. In determining whether the proposed recommendations are appropriate for Halifax day care centres, the question "Do Halifax day care centre populations differ from the populations surveyed in the literature and would this be the factor responsible for the lower CMV prevalence?" arises. Factors such as the socioeconomic status, day care centre population size and age range of the children in attendance were compared between this study and the reported literature to determine whether identifiable differences exist.

a. SOCIOECONOMIC DESCRIPTION

In the literature day care centres surveyed for CMV prevalence were classified on the basis of socioeconomic status of the day care centre itself (Pass et al 1982, 1984, Hutto and Pass 1984, Hutto et al 1985 A & B). The Halifax day care centres could not be classified according to the socioeconomic

description of the geographic area of the centre. Low socioeconomic day care centres could not be identified. Due to the structure of the Department of Social Services assistance program, that is the children's families not the centre were subsidized, a low socioeconomic day care centre would be unlikely to be representative of a low socioeconomic population. This inability to classify the socioeconomic status of the day care centre prior to the demographic descriptor survey differed from the previous described studies. After all the information was collected and discussions were held with the day care centre directors, two aspects of day care centre use became apparent; that the parents chose a day care centre that was convenient, either near the family's home, place of the parent's employment or to a centre where there was space available for the child and that the socioeconomic description of the day care centre population would have to be determined by the survey questionnaire sent to the children's parents.

The subset of Halifax day care centres included in this survey were situated in a middle socioeconomic geographic area; the data collected from the parents also indicated an overall middle class population. However, children from all socioeconomic divisions were found to be present in the day care centre populations. Populations surveyed in Pass et al's 1982 and 1984 surveys of children attending day care centres in Alabama, United States described the day care centre to have a

middle socioeconomic status. Adler (1985A) also surveyed a middle socioeconomic status day care centre population in a Virginian day care centre; however, the day care centre population consisted only of children whose parent(s) were hospital staff - a select group.

Although the surveyed Halifax day care centre populations reflected a middle socioeconomic status, children from all socioeconomic divisions were present. The CMV urine excretion prevalence was found to be lower than the reported prevalences. General population Halifax children within the same age range should have a higher CMV prevalence than the day care centre populations due to the different CMV prevalences found within the different socioeconomic segments throughout the general population (reviewed earlier). Therefore, the mixed socioeconomic status of the children attending the subset of Halifax day care centres was not responsible for the lower CMV excretion prevalence.

b. SIZE OF THE DAY CARE CENTRE POPULATIONS

The size of the Halifax day care centre populations were not of the same range as those studied in the United States. The United States CMV excretion prevalence surveys of day care centre populations ranged in number of children from 23 to 75, the mean size of the day care centre populations was 56 children (Table 18). Only one Halifax day care centre

TABLE 18 Reported day care centre enrollment.

The day care centres used for CMV excretion prevalence surveys were compared to determine the number of children in attendance and the age range of children that the day care centres contained.

AUTHORS	LOCATION	NUMBER OF CHILDREN	AGE RANGE
PASS ET AL 1982	ALABAMA USA	75	3 - 65 MOS
HUTTO ET AL 1985A	ALABAMA USA	68	1 > 48 MOS
		58	1 > 54 MOS
		62	12 > 48 MOS
HUTTO ET AL 1985B	ALABAMA USA	59	0 > 48 MOS
		58	13 > 48 MOS
		50	0 > 48 MOS
		51	13 > 48 MOS
		23	13 > 48 MOS
ADLER 1985A	VIRGINIA USA	66	3 - 60 MOS
MURPH ET AL 1986	IOWA USA	48	3 - 73 MOS
GRILLNER 1988 & STRANGERT	UNDEFINED SWEDEN	16	12 - 84 MOS
		15	12 - 84 MOS

day care centre surveyed contained over 60 children, the remaining centres were found to contain between 17 and 45 children. As previously stated in the Materials and Methods, most of the day care centres in the Halifax area accept between 10 to 19 children (although the mean number of children attending was calculated to be 30 children per day care centre). Therefore, for at least the reported United States day care centre populations surveyed, the United States day care centres consisted of larger numbers of children than the Halifax day care centres.

In Sweden the reported day care centre populations consisted of between 15 and 16 children. These smaller numbers of children in attendance are similar to the day care centre sizes found in Halifax (Grillner and Strangert 1988). Both Canadian and Swedish day care centres have to meet regulations for child space allotment: a minimum area per child in attendance. The Halifax day care centres are required to have a space allotment of 30 square feet per child in attendance. Grillner and Strangert (1988) did not state the regulation space allotment minimum per child.

The smaller number of children attending the individual day care centres may be responsible for the lower CMV excretion prevalence and transmission found with the Halifax day care centre populations. The higher CMV excretion prevalences in United States children may be due to the

presence of an increased opportunity to encounter many other children. Because of the large numbers of children found in the individual United States day care centres are larger there would be a greater chance of contacting a child excreting CMV.

c. AGE CLASSIFICATION

The formats of day care centres can usually be described in two classifications schemes. Either the children were segregated into smaller groups based on the child's age or the day care centre consisted of a common area for all the children. The British and United States day care centres were separated into individual classrooms for the different age groupings (Pass et al 1982, 1984, Hutto et al 1985A & B, Nelson et al 1987). The United States researchers utilized, for the most part, the same classifications as the day care centres. The Swedish day care centres studied by Grillner and Strangert (1988) did not separate the children into age groups, the children were tended in a common area. Each research group used a different age classification system to categorize the day care centre children. In some cases, the age categorization was based on the day care centre's system and, in other cases, the researchers divided the children into convenient age groupings.

The Halifax day care centres surveyed during this study consisted of both types of day care centre formats. Day care centres A, B, and C divided the children into major age divisions. Day care centres D and E did not separate the children on the basis of age, but rather, had common areas for all ages of children.

Grouping the children into individual age categories, as opposed to caring for children without regard for age, did not seem to influence the CMV excretion prevalence. Statistical analysis found no significant differences in CMV prevalence between these types of Halifax day care centres.

Age was a factor in the CMV excretion prevalence in Adler's (1985A) survey of Virginian children attending a day care centre in which a high transmittance of CMV occurred. Approximately half the children attending the day care centre attended day care since infants. A large number of children were also found to excrete CMV in Adler's 1988 study, 38 children younger than 36 months of age acquired CMV infections. Canadian day care centre populations exhibit decreased numbers of infant aged children attending day care centres. Very few day care centres in the Halifax area will accept children under the age of one year (Private communication, Nova Scotia Department of Social Services, 1984). This may be a factor to explain why the Halifax day care centre children had low CMV excretion prevalences.

**B. RISK CATEGORIES FOR CYTOMEGALOVIRUS EXCRETION IN HALIFAX
DAY CARE CENTRE CHILDREN**

Risk factors for CMV prevalence have been previously identified in the literature. During this study of CMV urine excretion prevalence and transmission within day care centre populations, data regarding demographic descriptors were collected, including information regarding the age of the children, family size and socioeconomic status, as well as, the age of the parents. The socioeconomic status of the families was based on two of the demographic descriptors: the education and occupation of the child's parents. Factors, such as socioeconomic status and age, which have been previously identified to influence the acquisition of CMV infection (Griffiths et al 1985) were included on the forms. Statistical analyses were performed on the collected data to determine whether any risk factors for the excretion of CMV could be identified within the surveyed populations.

When the demographic descriptors for the day care centre children excreting CMV were compared with those for the children not excreting CMV, no risk factors could be identified for CMV excretion. However, given that this population was not representative of the general population, but rather a population consisting of children aged from eighteen months to under six years from middle socioeconomic families attending day care, this result is not

unreasonable.

Pass et al (1982) could not identify any significant risk factors for prevalence of CMV excretion in day care centre children from middle to upper socioeconomic families. The following descriptors were examined for a possible correlation with CMV excretion; number of children, child's age, child's sex, age at entry into the day care centre, months of enrollment in the day care centre, breast feeding, number of siblings, parent's mean age, parent's mean number of years of education, and parent's occupation (Table 19). The only factor to be identified as a possible risk factor for CMV infection in the day care children was the transfer of a child from the infant class to the toddler class. The child has recently become ambulatory allowing close contact with other children, as well as, with CMV contaminated surfaces. Hutto et al (1985A) confirmed the observation that the usual CMV risk factors of socioeconomic background, race and breast-feeding history were not significant in the prevalence of CMV excretion within day care centre children. In fact, the only difference that was attributed to the increased CMV excretion prevalence was day care centre attendance.

TABLE 19 Reported day care centre population characteristics compared to the surveyed Halifax day care centre children.

The described demographic factors for Pass et al's reported day care centre populations were compared against those obtained for this study. Many similarities can be found among the day care centre populations.

	PASS ET AL 1982	PASS ET AL 1984	PASS 1985	OUR SURVEY 1984-1987
NO OF CHILDREN	70	103	188	139
AGE	3-65 MONTHS			12-60 MONTHS
M/F	37/33	53/50	98/90	65/72
NO OF SIBLINGS				
0	36	52	87	68
1	32	46	66	49
>2	2	5	0	15
PARENT AGE (YEARS)				
MOTHER	29+3.4	29.9+3.3	30.7	29
FATHER	32+4.3	32.1+4.2	34.4	32
EDUCATION (YEARS)				
MOTHER	16.7+2.1	16.2+2.2	16.5	POST HIGH SCHOOL
FATHER	15.7+2.5	16.2+2.9	16.7	POST HIGH SCHOOL
OCCUPATION	MOSTLY PROFESSIONAL	MOSTLY PROFESSIONAL	MOSTLY PROFESSIONAL	MOSTLY PROFESSIONAL

C. THE MOLECULAR EPIDEMIOLOGY OF CYTOMEGALOVIRUS WITHIN DAY
CARE CENTRE A

CMV transmission was directly assessed by restriction endonuclease analysis of the CMV genome rather than by indirect assessment by serological evidence. The progression of the transmission for CMV infection within the day care centre population, therefore, was able to be identified. CMV isolates which are nonepidemiologically related were identified by their unique electrophoretic fragment patterns; whereas, CMV isolates with identical restriction endonuclease electrophoretic patterns were considered to be epidemiologically related (Huang et al 1980A & B). Epidemiological relatedness of the viral isolates was assessed by use of three or more restriction endonucleases. If two isolates were then found to be epidemiologically related, the results obtained from two additional restriction endonuclease analyses (for a total of five restriction endonuclease analyses) were assessed prior to determining the two isolates were epidemiologically related. CMV isolates found to be identical by restriction endonuclease analysis can be said to be the same strain of CMV. By this criterion, CMV was found to be transmitted amongst the day care centre A children or by another common source.

As previously stated, in order to identify the actual

transmission progression, susceptible individuals (individuals not excreting CMV) were identified within the population. If the entire day care centre population, staff and children, were able to be surveyed for presence of CMV and the CMV isolates obtained were subsequently identified by restriction endonuclease analysis, then the actual progression of CMV transmission would be able to be identified. These stringent requirements usually can not be met during a CMV survey such as this study. The inability of this survey to comply to these requirements included the following reasons: uninterested parents, parents refusing to give consent to allow their children to participate in the survey, children leaving the day care centre prior to providing a specimen, children refusing to provide a specimen and day care centre staff refusing to participate in the survey.

The limitations of a molecular epidemiological survey of CMV transmission within children attending the day care centres must take into account the inability of the study to meet these stringent requirements. Given the limitations of the study, the following statements on CMV transmission within the day care centre children may be made. No definite progression of CMV transmission may be identified for two or more children concurrently excreting the same CMV strains unless one child had had a previously negative urine for CMV. One child (or more, dependent on the cluster size)

can be identified as having been infected within the day care centre environment; however, the identity of which child specifically acquired the infection would not be able to be determined. The remaining child may have either acquired its infection external to the day care centre or possibly from an unsurveyed day care centre child or staff member.

A partial chain of transmission may be determined if two children, one previously identified as excreting the strain of CMV and the other as being susceptible to infection by that strain, are found to be excreting the same strain of virus. The newly infected child can be identified as being epidemiologically related to the CMV-excreting child. The CMV infection can be identified as occurring as a result of the day care centre environment, providing the children are not siblings and have no social contact except as a result of day care centre attendance. However, because not every child and staff member were examined for CMV presence, the statement that the CMV-excreting child was the source of infection can not be made. The possibility exists that a staff member or third unsurveyed child was the source; however, an epidemiological link would exist between any children or staff member excreting that particular strain of CMV. CMV transmission among the children attending the surveyed Halifax day care centres was found to be a rare event.

Two unrelated children attending the same day care centre A group were found to be concurrently infected by the same strain of CMV (DC 14 and DC 21). An epidemiological relationship resulting from viral transmission between the children, or through a third unsurveyed child or staff member is indicated. The progression of the transmission of CMV could not be determined because the two children were found to be concurrently excreting CMV during the first survey in December 1984. Neither child had previously been found to have a CMV negative urine specimen. It is known, however, that both children attended the same day care centre group, the junior group, so the opportunity for contact and viral transmission existed between these two children.

During the 1984 survey of day care centre A, a pair of children attending different day care centre groups had similar but not identical strains of CMV (DC 01 and DC 15). In the 1985 survey a third child (DC 13) was identified as newly infected with the same CMV strain previously present in the child excreting DC 15. Because the newly infected child excreting DC 13 had been previously found to be negative for CMV presence and the two children (DC 15 and DC 13) were concurrently excreting the same CMV strain in 1985, day care centre transmission of CMV was identified to have occurred. Although both these viral isolates were found to

have similar electrophoretic fragment patterns (varying from 1 to 2 band differences with the enzyme *Bam*HI to many differences with the enzyme *Kpn*I) to DC 01, DC 01 is not considered to be the same CMV strain. CMV was transmitted in the day care centre environment to the newly infected child (DC 13), most likely by the child found to be positive in 1984 (DC 15) or possibly via a third unsurveyed CMV-excreting person. As previously stated due to lack of parental consent, all the children attending day care centre A were not surveyed for CMV presence. For this reason the statement that the child excreting DC 15 infected the child excreting DC 13 can not be made. However, the viral electrophoretic fragment patterns following restriction endonuclease digestion of the isolated CMV genomes were identical so the viruses can be identified as epidemiologically related. Because day care centre attendance in Halifax was found, for the most part, not to be related to the family's home location, the children would not have had contact with each other except through day care centre attendance (the home addresses for these two children confirmed this fact). For these reasons the transmission of CMV can be said to have occurred as a result of day care centre attendance.

The two previously mentioned similar but not identical CMV strains, DC 01 and DC 13, were isolated from children who are siblings. Given that the viral restriction

endonuclease fragment patterns are quite similar, three hypotheses may be postulated for the similarity of these virus isolates. The first hypothesis is that the viruses are coincidentally very similar and no viral transmission occurred between the siblings. The second hypothesis is viral genetic variation occurred in one of the strains of CMV and that viral transmission occurred between the siblings (Huang et al 1980 A & B). The third hypothesis is that the child excreting DC 01 may in fact be superinfected with two strains of CMV accounting for the difference in the band patterns and again, virus transmission had occurred (Adler 1985A). However, it is unlikely that the child excreting DC 01 infected its sibling (DC 13). The virus (DC 13) would have had to revert back to a strain with the restriction enzyme digestion electrophoretic fragment pattern of DC 15 or both CMV isolates (DC 13 and DC 15) would have had to undergone the same genetic variation. If, in fact, the child excreting DC 01 did infect the child excreting DC 13, one would more likely expect either an identical electrophoretic fragment pattern to DC 01 or a further variation to occur rather than a reversion to the DC 15 pattern. The viral isolates were not plaque purified prior to restriction endonuclease analysis so if a case of superinfection (hypothesis 2) was present then it could not be easily identified. Unfortunately very few studies have been published on CMV superinfection as identified from specimens from a single site; for example, urine. Most

methodologies for restriction endonuclease analysis do not include plaque purification steps to identify whether more than one strain of CMV is present.

Two other viral isolates from day care centre A, DC 30 and DC 33, were found to be similar in the restriction endonuclease patterns, but not identical. The restriction endonucleases digestions for these two isolates varied from few (one to two band differences) to many band differences. Again the previous three hypothesis would apply.

An interesting situation was found to exist in day care centre C, a pair of siblings attended the centre. One child was found to be positive for CMV excretion and the other sibling did not excrete virus. CMV transmission neither occurred between the siblings within the day care centre environment nor home environment. The possibility that the sibling not excreting CMV may have had a previous infection and ceased shedding the virus in urine does exist; however, the age of the uninfected child makes this possibility unlikely. The infected sibling was four years of age; whereas, the uninfected child was three years old.

Shedding of CMV in the urine for an extended period of time has been previously described. (Weller et al 1957, Faix 1985). During the course of this survey, five children were found to excrete CMV in their urine over a period of from

two to three years (DC 01, DC 14, DC 15, DC 21, and DC 37). In each case the original virus isolate was reisolated in each of the subsequent specimens, as determined by restriction endonuclease electrophoretic fragment patterns of the viral DNA. The child excreting DC 14 was the only child found to stop shedding CMV into its urine. The virus ceased to be shed after the second specimen. No child was found to be reinfected or superinfected by a different CMV strain as defined by a change in the restriction endonuclease electrophoretic fragment patterns in successive viral isolates.

D. IDENTIFIABLE STRAINS OF CYTOMEGALOVIRUS

During the study of Halifax day care centre populations no CMV strain was found to predominate in the day care centre children as was found by Adler (1988). CMV strains were identified as CMV isolates with unique electrophoretic fragment patterns following restriction endonuclease analysis of the DNA from the isolate. CMV isolates from different children found to have identical electrophoretic fragment patterns were considered to be the same CMV strain and, thus, epidemiologically linked. In contrast Adler's study (1988) of a total of 108 children attending a Virginian day care centre, surveyed over an extended time period, identified 32 children excreting the same CMV strain (26 children all under the age of three began excreting the virus during the 26 month survey period). Two other groups with common isolates were also identified, one consisting of seven children and the other of five children. Epidemiological relationship analysis was performed using the restriction endonucleases *Bam*HI and *Eco*RI. The day care centre population was large consisting of approximately 63 children, a large number of these children had been enrolled in the day care centre before the age of six months. None of the other day care centres described in the literature showed the predominance of one CMV strain (Hutto and Pass 1984, Grillner and Strangert 1986, Murph et al 1986, Nelson et al 1987). The Halifax day care centre population

differed from the population described in Adler's survey. The enrollment of the Halifax day care centres was generally smaller than that described in Adler's study and the large number of children under the age of three found in the Virginian day care centre was not seen in the Halifax day care centre populations. As previously described very few infants attend Halifax day care centres; in fact, the majority of children are above the age of 30 months. These factors may have influenced the transmission of a CMV strain such that large numbers of children excreting a single CMV strain were not found.

The stability of the strain distribution in the Halifax day care centre populations was not assessed due to the lack of a predominating CMV strain. The CMV strains from children excreting CMV over extended time periods (up to three years) were identified in each case to be the same strain as originally isolated. The CMV strains, isolated from DC 14 & DC 21 and DC 13 & DC 15, were found to be excreted for extended periods of time. However, either the CMV strains found in the Halifax day care centre children were not very infectious or transmission opportunities did not occur with the same frequency found in the day care centre surveyed by Adler (1988).

V. CONCLUSIONS

Previous studies reported in the literature have found the CMV prevalence within day care centre populations to be higher than expected (Pass et al 1982, 1984, Hutto et al 1985B). A survey was undertaken to determine if the high CMV prevalence reported within the day care centre populations similarly occurred in Halifax, Nova Scotia, Canada day care centre populations and to determine if the recommendations proposed for United States day care centre populations were appropriate for Halifax day care centre populations. Five day care centre populations were surveyed for presence of children excreting CMV in their urine.

During the survey CMV was found to be excreted by a total of 10 children with the CMV prevalence ranging from 0% to 33% in different centres. The overall CMV prevalence within the combined populations of children attending day care centres was 13%, 95% confidence interval from 6% to 21%. In the repeated sampling of day care centre A, a total of 13 children were found to excrete CMV. The CMV excretion prevalence was 15% for the 1984 survey, increasing to 26% during the 1985 survey and decreasing back to 15% for the 1986 survey. The variation was found not to be statistically significant. Two children attending day care centre A excreted the virus over a three year period. When

the statistical analysis was performed on both the data from day care centre A and the total data from all the children assessed for CMV excretion, no risk factors could be identified.

The CMV excretion prevalence in the day care centre populations was found to increase with age from 18 months to 41 months. A decline in CMV excretion was found during the ages of 42 months to 60 months. Children between the ages of 32 to 41 months were most likely to excrete the virus. This age of increased CMV excretion prevalence is older than that found in United States populations. Very few infants attend Halifax day care centres. In contrast, many reported United States day care centre population have a large infant component. The fact the Halifax day care centre populations tend to be older at the age of entrance to a day care centre than those reported for the United States may explain the older age when increased CMV excretion prevalence is seen.

CMV transmission among the children occurred within day care centre A; however, transmission was found to be a rare event. At least two children were infected with CMV strains already present within the day care centre population. No strain of CMV was found to predominate among the day care centre children. These results were in contrast to the findings of Adler (1988) in his survey of a Virginian day

care centre. A high level of CMV transmission (although the transmission was described as "slow") was found to occur as seen by a large number of children (32) excreting the same CMV strain, 26 of the children commenced excretion of the virus while in attendance at the centre. Older age at day care centre entrance and smaller day care centre enrollment in the Halifax centres may be the factors responsible for the low CMV transmission among the Halifax day care centre children.

The CMV prevalence in the Halifax day care centre children and CMV transmission results agree with Grillner et al's (1988) conclusion that CMV is not extremely infectious, as determined by the low amount of transmission among the day care centre children. The CMV excretion prevalence found in Halifax day care centre populations was lower than that reported for United States day care centre populations. Because transmission was found to occur even though it was a rare event, the recommendation that pregnant women encountering day care centre children should prevent contact with children's body fluids by practicing good hygienic measures is warranted.

The limitations of this study include the following factors; geographic location - Halifax, day care centre population, and middle socioeconomic class status population. The day care centres in the survey did not

exhibit major differences in socioeconomic status; although, all socioeconomic divisions were represented. According to the Nova Scotia Department of Social Services, Day Care Center Divisions' classification system, major differences in the socioeconomic status of the Halifax day care centres should not occur. Therefore, the CMV prevalence for the children attending the five day care centres would not be generalizable to the Halifax children population, but should be able to be generalized to children attending Halifax day care centres.

As previously stated this study population consisted of a select group of children, children attending day care centres, the CMV prevalence would not reflect the overall CMV prevalence in the Halifax area children. Based on previous studies of CMV epidemiology, the CMV prevalence results for the general Halifax children population should be lower due to the decreased contact with other children and the influence of the population's socioeconomic status.

APPENDIX I

DEFINITION OF EPIDEMIOLOGICAL TERMS (condensed from Table
4.1 in Halsey 1986)

Epidemiology - the science concerned with defining and explaining the interrelationships of factors that determine disease and health frequency and distribution in populations.

Incidence - the number of "new" cases of disease within a specific period in a defined population. The population must contain individuals free of the disease at the study origin.

Incidence Rate - the number of new cases of disease divided by the population at risk in a specified period.

Prevalence - the number of cases of a disease in a specified population that exist at a specific instant of time or during some time period, usually given as a "period prevalence". (i.e. the number of persons affected during a specified period. The number is influenced by the incidence and duration of disease.)

Prevalence Rate - the prevalence divided by the population.

Confounding - extraneous factors influencing the results of a survey.

APPENDIX II

The IZAAK WALTON KILLAM
HOSPITAL for CHILDREN

5850 UNIVERSITY AVENUE • P.O. BOX 3070 • HALIFAX, N.S. • B3J 3G9



Dear Parents:

We are a Dalhousie University research team conducting a study on virus prevalence within Day Care Centers. This project has been sanctioned by both the Department of Public Health (Cobequid Health Unit) and the Department of Social Services (Day Care Services), and is funded by a grant from Health and Welfare, Canada.

We would like to have your child participate in this project. We require a small amount of urine which will be collected by the Day Care Center staff at the Day Care Center. There will not be any expense to you. If you should decide to allow your child to participate in the study but feel you do not wish to fill out the survey form, please indicate this choice. The decision to allow your child to participate in the study or to withdraw your child from the study will in no way affect the child's care. Any personal information will be kept in strict confidence.

If you have any questions on this project or on your child's participation, please contact me and I will be happy to answer these questions. My telephone number at the Izaak Walton Killam Hospital for Children is 428-8491.

Please aid us in this study, we hope that it may be of benefit to young children.

Sincerely;

Valerie Mann
Jaqueline

Valerie Mann M.Sc.
Department of Microbiology
Dr. J. Embil,
Departments of
Microbiology,
Community Health and
Epidemiology
and Pediatrics, Dalhousie
University

Day Care Center Attending _____

Information about child:

Name _____ Age _____ Sex _____

Address _____

Information about parents:

Mother's Name _____ Age _____

Education _____

Occupation _____

Father's Name _____ Age _____

Education _____

Occupation _____

Other Children in the Household 1 _____
and their ages 2 _____

3 _____

4 _____

5 _____

6 _____

I will / will not allow my child to take part in this study

Signature _____

Date _____

Day Care Center Consent Form

I will/will not allow a urine specimen to be collected from my child for a study on virus prevalence within day care centers. I understand there will be no cost to me and any personal information will be kept in strict confidence.

Signature _____

Date _____

APPENDIX III

- 1) Basal Medium Eagles (Diploid)
 Earles' salts
 L-glutamine
 without sodium bicarbonate

- 2) Phosphate Buffered Saline (PBS)
 sodium chloride 80.0 g
 potassium chloride 2.0 g
 sodium phosphate dibasic 20.9 g
 potassium phosphate 2.0 g
 per 10 l H₂O

- 3) 0.25% Trypsin
 trypsin 2.5 g
 sodium chloride 8.0 g
 potassium chloride 0.2 g
 sodium phosphate dibasic 1.5 g
 potassium phosphate monobasic 0.2 g
 phenol red (10X) 1.0 ml
 dextrose 0.5 g
 per 1 H₂O

- 4) HIRT Buffer
 Sodium Dodecyl Sulfate (SDS) (Bio-rad) 0.6 g
 TRIS 1.0 ml
 EDTA 0.37224 g
 per 100 mls H₂O; pH=8.0 (HCl)

- 5) Saturated Phenol
 Phenol 20.0 ml
 NaCl .075 mol/l
 EDTA (pH 8.0) .05 mol/l

- 6) Dialysis Buffer (DB)
 TRIS 302.75 g
 NaCl 145.90 g
 EDTA 18.52 g
 per 1 H₂O; pH=8.0 (NaOH)

- 7) BamHI Buffer
 TRIS-HCL (pH 8.0) 20.0 mM
 sodium chloride 100.0 mM
 magnesium chloride 7.0 mM
 2-mercaptoethanol 2.0 mM

- 8) HindIII, XhoI (CORE) Buffer
 TRIS-HCL (pH 8.0) 50.0 mM
 magnesium chloride 10.0 mM
 sodium chloride 50.0 mM

- 9) KpnI Buffer
 TRIS-HCL (pH 7.5) 6.0 mM
 sodium chloride 6.0 mM
 magnesium chloride 6.0 mM
- 10) SalI Buffer
 TRIS-HCL (pH 7.6) 8.0 mM
 sodium chloride 150.0 mM
 magnesium chloride 6.0 mM
 Na₂EDTA 0.2 mM
- 11) Tris-Acetate Buffer (TEA)
 TRIS 121.13 g
 Sodium acetate 54.40 g
 EDTA 14.9 g
 NaCl 23.38 g
 per 1 H₂O; pH=8.05 (acetic acid)
- 12) Stop Buffer
 EDTA (0.2 M) 0.745 g
 Bromo Phenyl Blue (0.1%) 0.01 g
 Sucrose 6.0 g
 per 10 ml H₂O; pH=7.5 (NaOH)
- 13) Modified HIRT Buffer
 TRIS .02 M
 EDTA .002 M
 SDS 1.2 g
 per 100 ml H₂O; pH=7.6
- 14) TRIS-EDTA Buffer (TE)
 TRIS 2.42 g
 EDTA .744 g
 per 100 ml H₂O; pH=7.6
- 15) TRIS-phosphate Buffer (TPE)
 TRIS 270 g
 85% Phosphoric acid 38.8 ml
 EDTA 100 ml
 per 1 H₂O; pH=7.8

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