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Detection of Neonatal Calf Diarrhea Virus (NCDV) and Human Infant Reolike Diarrhea Virus

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DETECTION OF NEONATAL CALF DIARRHEA VIRUS (NCDV) AND HUMAN INFANT REOLIKE DIARRHEA VIRUS

by

Martin Wyatt Peterson

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Martin Wyatt Peterson

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ABSTRACT

Detection of Neonatal Calf Diarrhea Virus (NCDV)

and Human Infant Reolike Diarrhea Virus

by

Martin Wyatt Peterson, Master of Science

Utah State University, 1975

Major Professor: Dr. Rex S. Spendlove
Department: Biology

The purpose of this study was to develop a diagnostic test and conduct a survey for the neonatal calf diarrhea virus (NCDV) and human infant reolike diarrhea virus. Two immunologic methods were developed in this investigation.

Immune electron microscopy (IEM) and the fluorescent viral precipitin test (FVPT) are the methods used to detect NCDV and the human virus. Both methods are based upon the principle that viral aggregates form when the virus is reacted with anti-NCDV antibody. Aggregates in the IEM method are negatively stained and observed with the use of an electron microscope. Fluorescein labeled antibody is used in the FVPT and the resultant aggregates are observed with the use of epi-fluorescence microscopy.

IEM and the FVPT are sensitive, specific and rapid. The sample is examined within two hours after arriving at the laboratory. Both methods are useful; however, the FVPT allows for more general application because it requires less technical skill and relatively inexpensive equipment is used.

NCDV was found in 41% (14/34) of diarrheic calves tested in northern Utah and NCDV antibody was in 100% (16/16) of adult animals tested. The human reolike virus was found in 80% (4/5) of the infants tested.

(47 pages)

INTRODUCTION

In March of 1969, Mebus et al. (1969) reported isolation of viral particles from a fecal specimen of a calf in north central Nebraska. Since that time, the virus has become known as the neonatal calf diarrhea virus (NCDV). It has been isolated in cattle herds in various parts of the world (Turner, Caple, and Craven, 1973).

Detection of NCDV depended on observation of fluorescent antibody stained cells that had sloughed from the calves' small intestine, or on direct electron microscopic identification of the agent. These methods were either very time consuming or unreliable. Recently, Barnett et al. (1975) developed an immunofluorescent cell technique that is partially effective in identifying infectious NCDV particles from fecal material.

The purpose of this investigation was to compare the fluorescent virus precipitin test (FVPT) (Foster, Peterson, and Spendlove, 1975), with immune electron microscopy (IEM), and to determine the prevalence of NCDV and its related human virus in northern Utah. Using the IEM adenovirus detection technique of Vassall and Ray (1974), optimal conditions for detection of NCDV, the human reolike virus, and their respective antibodies were determined.

LITERATURE REVIEW

History

General

Diarrhea of calves, or calf scours, has been a problem in the beef and dairy industries for many years. Morbidity may reach 90-100% which may result in stunting of growth or even death of the animal. In 1925, Smith and Orcult (1925) recognized that an infectious agent caused some of the disease. Escherichia coli was incriminated, but it has been difficult to consistently produce experimental E. coli infectious with clinical symptoms (White, Mebus, and Twiehaus, 1970). A severe outbreak of scours occurred in western Nebraska in 1966 and the state legislature commissioned a group from the University of Nebraska to study the problem. The study was begun in 1967 (Anonymous, 1973). Mebus et al. (1969) obtained fecal specimens from herds in north central Nebraska. Filtrates from these specimens produced diarrhea in gnotobiotic calves. An anti-NCDV fluorescent antibody enabled Mebus et al. (1969) to observe fluorescing epithelial cells in the fecal material.

Nature of disease

Mebus et al. (1969) reported that a calf appearing normal at night could be depressed and have watery, yellowish feces in the morning. The amount of mucus present in the feces would gradually increase, the temperature range would be 38.8-39.7 C, and dehydration would occur. With the use of gnotobiotic calves, it was shown (Mebus, 1972) that an

incubation time of 13-14 hours is required before the virus is produced in the villous epithelial cells of the small intestine. Stair et al. (1973) found that NCDV is produced within the cisternae of the endoplasmic reticulum of the villous epithelial cells. The epithelial cells begin sloughing within 5-6 hours after the diarrhea begins and are replaced by cubodial cells. A decrease in digestion and absorption are responsible for the persistent diarrhea. The calf develops a degree of dehydration, electrolyte imbalance, and starvation. Bacteria proliferate, due to increased nutrients available, and bacteremia results (Mebus, 1972). White, Mebus and Twiehaus (1970) found that E. coli is the predominant organism found in bacteriologic examination of feces. When bacteremia results, E. coli is found in the liver, kidney, spleen, and joint cavities. Gnotobiotic calves infected with NCDV and E. coli have a more severe infection than with only NCDV (Stair, et al., 1973).

Characteristics of the virus

Welch (1971) reported the virus to have a density of 1.359 in CsCl and a genome of ribonucleic acid (RNA). The particle has a diameter of 64 nm and the hexagonal core of 36 nm diameter. Welch and Thompson (1973) described NCDV as not sensitive to ether or chloroform, acid pH stable, and relatively thermostable.

Diagnosis of Viral Infection

Fluorescent antibody technique

Mebus et al. (1969) were the first to report the use of fluorescent labeled antibodies in the detection of NCDV. The antigen was

obtained from calf feces and the antibody produced in rabbits was labeled with fluorescein isothiocyanate (FITC). The virus was detected by either staining frozen thin sections of the small intestine or by staining smears of intestinal or fecal material and observing the fluorescent infected intestinal epithelial cells. When using tissue culture techniques, Welch and Twiehaus (1973) found that 5 days were needed for maximal development of the antigen.

Barnett et al. (1975) developed a quantitative fluorescent antibody method using Madin Darby bovine kidney (MDBK) cells. This technique enabled limited detection of modified or wild-type NCDV and the corresponding antibody.

Immune electron microscopy (IEM)

As early as 1941, Anderson and Stanley (1941) combined tobacco mosaic virus (TMV) with TMV antisera and observed aggregates of the virus in the electron microscope. In 1967 Best et al. (1967) demonstrated a pleomorphic particle that was found to be the rubella virus. Since that time many viral agents have been found to form aggregates when incubated with homologous antibody. Some of these are echo, coxsackie and polio viruses (Anderson and Doane, 1973), coronavirus (Kapikian et al. 1973), Australia/SH antigen (Kelen, Hathaway, and McLeod, 1971), and rhinoviruses (Kapikian, Almeida, and Stott, 1972).

The method of Best et al. (1967) was the first practical IEM method developed. The antigen and antibody were mixed, incubated and spun at a moderate speed to pellet the viral aggregates. The supernatant fluid was removed, and the pellet was resuspended in water and

phosphotungstic acid stain (PTA). The suspension was then placed on a grid and observed.

Other methods have been developed that do not require the centrifugation step. The antigen and antibody are mixed, incubated, and a drop of this mixture is placed on an agar surface. A grid is placed on the drop and removed when the liquid diffuses into the agar (Kelen, Hathaway, and McLeod, 1971). Microtiter plates have replaced test tubes in two IEM tests. Anderson and Doane (1973) mixed agar and antisera in microtiter wells and placed a grid on the agar surface. The virus suspension was then added to the well, allowed to dry, and the grid was removed and observed. By mixing antigen and antisera in a microtiter well, incubating it, dipping a grid in the well, staining and observing, Vassal and Ray (1974) developed a rapid and quantitative test.

The antigen-antibody reactions of IEM have been shown to have a high enough degree of specificity that the technique is being considered as a routine typing system for virus groups. Some of the virus groups that have been tested are adenoviruses (Vassall and Ray, 1974), coronaviruses (Kapikian et al., 1973) and eleven different enterovirus serotypes (Anderson and Doane, 1973).

IEM has been shown to be a sensitive method for detecting antigen and antibody. Kelen, Hathaway, and McLeod (1971) found that, in detecting antigen, IEM has an overall positivity rate equal to the complement fixitation test (CFT) and higher than that of the crossover electrophoresis test (COEF). In detecting antibody, IEM is more sensitive than the CFT or COEF. They also demonstrated that with excess

antibody, IEM gave positive results but the CFT gave negative or equivocal results because of the prozone phenomenon. When comparing IEM with conventional electron microscopy, Anderson and Doane (1973) reported that IEM is 100 times more sensitive in detecting some of the enteroviruses.

In quantification, IEM has been shown to be an effective method. Anderson and Doane (1973) demonstrated that the number and size of aggregates becomes smaller as the serum reaches its endpoint where no more aggregates form.

Almeida and Waterson (1969) stated that if the antibody is slightly greater in ratio than the antigen, the aggregates reach maximum size. Several investigators have stated as did Kapikian et al. (1972) that "heavily coated particles were usually found to be in small aggregates, whereas those with less antibody usually formed larger aggregates." Lafferty and Oertelis (1961) explained that neutralization is in two stages. The first a formation of a "freely dissociable union" and the second a stabilization stage. When antibody is in great excess, the stabilization step proceeds slowly because of a lack of second combining sites and no or very few virus aggregates are formed.

Hemagglutination and plaque formation with NCDV

No hemagglutination with NCDV has been observed (Welch and Thompson, 1973).

Welch and Twiehaus (1973) reported that plaque formation by NCDV could not be reproduced consistently and was generally unsuccessful.

Epidemiology of NCDV

Incidence of NCDV

The NCDV has been demonstrated in cattle herds in the United States, Canada, Australia, and the United Kingdom (Woode and Bridger, 1974).

In field outbreaks of calf diarrhea in the United Kingdom, Woode and Bridger (1974) found that disease in 80% of the calves less than three weeks of age was caused by neonatal calf diarrhea reovirus (NCDR). NCDR, which is NCDV, was not isolated from five outbreaks of older animals. Ten percent of all calves in the United States are affected by scours (Anonymous, 1973).

Incidence of NCDV antibody

Woode and Bridger (1974) reported that all of the herds that they tested in the United Kingdom were serologically positive for the NCDV antibody. The normal antibody titer found in healthy cattle was 1:20 (Flewett et al., 1974), but this increased in calves to 1:640 after four weeks of infection. Through a personal communication, C. A. Mebus (1974) has reported that there is antibody to NCDV in some normal human sera. Piglets have been infected with NCDV, and they developed antibody to the virus (Wood and Bridger, 1974).

Relationship of Reolike Infantile Diarrhea Virus to Neonatal Calf Diarrhea Virus (NCDV)

Characteristics of the reolike virus

A type of gastroenteritis of infants and young children has been shown to be caused by a reolike virus similar to NCDV. Bishop et al.

(1973) have defined infant gastroenteritis as an illness of less than 10 days duration associated with diarrhea and vomiting, in which there was no evident cause other than the human reolike virus for the symptoms.

The diameter of the double shelled capsid of the reolike virus is 70-75 nm and the single shelled capsid has a diameter of 60 nm. The density of the single shelled particles is 1.36 g/cm^3 (Holmes et al., 1974). Bishop et al. (1974) described the reolike virus as one resembling the viruses of epizootic diarrhea of infant mice (EDIM) and calf scours (NCDV). The core has a diameter of 36-38 nm and the outer layer of capsomers has been described as giving the virus the appearance of a rim on a hub (Flewett et al., 1974).

Incidence of the reolike virus

Holmes and Ruck (1974) reported that 70-80% of the children hospitalized in Melbourne with acute gastroenteritis were infected by an orbivirus. The reolike infantile diarrhea virus has been called an orbivirus by several investigators. Bishop et al. (1974) found that the virus was as profuse or more so in fecal extracts as in the duodenal mucosa. The virus has been detected in the stools of children ranging in ages from 3 to 49 months with an average age of 15.8 months (Kapikian et al., 1974). Virus particle excretion is maximal during the third to fourth day of illness (Holmes et al., 1974), although an infected infant ceased to produce the virus on day four (Bishop et al., 1974) while others have excreted virus up to the tenth day after onset of symptoms. Flewett, Bryden and Davies (1973) stated that the particles appear to be more common at some times of the year. Sexton et al. (1974) reported the morbidity and mortality due to gastroenteritis in

aboriginal children are 10-15 times greater than in the white Australian population. The reolike virus has been found in children in the United States (Kapikian et al, 1974), the United Kingdom, Australia, Singapore, Canada and Rhodesia (Sexton et al., 1974).

Incidence of the reolike virus antibody

Bishop et al. (1973) speculated that "the age-range of children vulnerable to orbivirus may be similar to that for respiratory syncytial virus, against which all children have neutralizing antibodies by seven years of age." Of the infants having the disease, the antibody titer was low or absent in the acute stage, but all had significant antibody titers in the convalescent state (Kapikian et al., 1974). Holmes et al. (1974) reported that rabbit hyperimmune sera and human convalescent sera to the reolike virus caused IEM aggregation of the reolike virus as did anti-NCDV and anti-EDIM sera.

MATERIALS AND METHODS

Virus

One lot of neonatal calf diarrhea virus (NCDV) was obtained from Norden Laboratories, Lincoln, Nebraska. This virus was a vaccine strain produced in tissue culture in their laboratories.

The majority of NCDV used in this study was the wild type virus obtained from the feces of diarrhetic calves. These were supplied by Norden Laboratories, veterinarians and farmers in Cache County, Utah, and by the diagnostic laboratory, Department of Veterinary Science, Utah State University.

The human reolike virus was obtained from the diarrhetic stools of infants; some infants having been admitted to the Logan L. D. S. Hospital, Logan, Utah.

The reovirus was a Lang strain (type 1) that was propagated in this laboratory.

The parainfluenza virus type 3 (PI-3) was obtained from the Veterinary Science Department, Utah State University.

Virus Suspension Preparation

The fecal material was prepared by electron microscopic examination in the following manner. Liquid stool material was diluted 1:2 with phosphate buffered saline (PBS) or when the material was of a semi-solid consistency, it was diluted 1:4 with PBS prior to centrifugation. This material was mixed and then spun in a SS-34 rotor of a Sorvall

RC-2B centrifuge (Dupont Company, Sorvall Operations, Newtown, CT) at 3020 xg for 10 minutes. After centrifugation, the supernatant fluid was carefully drawn off with a Pasteur pipette. This supernatant layer was then filtered and/or tested.

When a fecal sample was filtered, the supernatant layer from the centrifugation step was passed through a 13 mm, 0.45 µm pore size Millipore filter (Millipore Corporation, Bedford, Mass.) held in a Pop-Top filter holder (Nuclepore Corporation, Pleasanton, Calif.).

Antisera

In experiments where the virus was the variable factor, lot R-2 NCDV antiserum produced in rabbits was supplied by the Veterinary Science Department, University of Nebraska. The serum specimens tested for NCDV antibody were obtained from convalescent calves diagnosed as having NCDV caused diarrhea or from adult animals at a local abattoir. The reovirus antiserum was obtained from a goat immunized against reovirus types 1, 2, 3. Mycoplasma arthritidis antiserum was produced in a pony.

Immune Electron Microscopy

Samples of the supernatant layer obtained after the centrifugation of the fecal material were used as the virus suspensions in all IEM procedures. A 0.05 ml sample of the fecal supernatant was mixed with 0.05 ml of antiserum in a V-shaped well of a microtiter plate (Microbiological Associates, Bethesda, Md.). To minimize evaporation, the plate was incubated in a humid atmosphere of a water bath set at 37 C.

Formvar (0.25% in ethylene dichloride) coated electron microscope grids (copper, 3 mm diameter, 300 mesh) were coated with carbon in a Model VE-10, Varian Vacuum Evaporator (Varian Vacuum Division, Portland, Ore.). The microtiter plate containing the incubated virus-antibody reactants was tilted slightly, and a carbon coated grid was lowered gently into and immediately removed from the sample containing well. The grid was then placed onto a droplet of 2% phosphotungstic acid (PTA) (adjusted to pH 7.0 with 5N KOH). This is known as the "grid on drop" technique. After staining, the grid was examined in a Carl Zeiss EM 9S-2A (Carl Zeiss Inc., New York, N. Y.) electron microscope at a plate magnification of 28,000X. Three grid squares were examined to detect the presence of viral aggregates. A positive IEM test requires detection of aggregates of three or more viral particles (Kapikian et al., 1972).

In using the method of Kapikian, Almeida, and Stott (1972), 0.9 ml of a dilution of antisera was mixed with 0.1 ml of a filtered (0.45 μm pore size) virus sample. This mixture was incubated for 1 hour at 20 C and then overnight at 4 C. The mixture was then centrifuged in a SS-34 rotor of a Sorvall RC2-B centrifuge at 17,300 xg for 1 hour. The supernatant fluid was aspirated with a pasteur pipette, the pellet resuspended in 0.1 ml of distilled water and 0.1 ml of a 3% PTA solution was added. This mixture was added to a grid for 30 seconds, after which it was then removed and the grid was observed.

The Kelen, Hathaway, and McLeod (1971) method consisted of adding 5 ml of a 0.8% ionagar (Colab Laboratories, Chicago Heights, Ill.) solution to a microscope slide. While the agar was solidifying, an

antibody dilution was mixed in a 1:1 ratio with a virus suspension. After a 2 hour incubation at 37 C, 0.01 ml of the virus-antibody mixture was added to the agar surface. A carbon coated grid was placed onto the reaction mixture. When the liquid had migrated into the agar, the grid was removed, and viral aggregates were negatively stained with 2% PTA and observed.

Fluorescent Antibody Procedures

A method described by Barnett et al. (1975) for detecting infectious NCDV by infecting Madin Darby bovine kidney cells and then staining with a fluorescein conjugated anti-NCDV antibody was used.

A FVPT has been developed by Foster, Peterson and Spendlove (1975). In this procedure, total NCDV particles are detected by incubating a virus suspension with a fluorescein labeled anti-NCDV antibody and then observing the resultant aggregates using an epi-fluorescence microscope.

Optimal Test Conditions

Using the Vassal and Ray (1974) IEM method and an incubation time of 1 hour, the optimal incubation temperature was determined by incubating samples of antigen-antibody mixtures respectively at 37 C, 20 C, and 4 C. Specimen grids were then dipped into the antigen-antibody mixtures, negatively stained and observed.

In determining the effect of shaking on aggregate size and number, a microtiter plate containing the antigen-antibody mixtures was placed on a gyrotory shaker, model G-76 (New Brunswick Scientific Company,

New Brunswick, N. Y.). Respective samples were incubated for 1 hour at 37 C at speeds of 95, 110, or 120 rpm. The mixtures were sampled, stained and observed by electrom microscopy.

To determine the optimal staining conditions, the electron microscope grids were stained in one of two ways: (1) the "grid on drop" method as described by Dawes (1971) or (2) the "drop on grid" technique (Hayes, personal communication, 1974).

In the "drop on grid" technique, the edge of a carbon coated grid was supported on a piece of double stick scotch tape. A drop of stain was placed on the grid and after the prescribed time, the drop was removed with a strip of absorbant paper. The grid was then observed.

A viral transport medium described by Leibovitz (1969) was used or modified and used to test for viral survival at refrigeration temperatures.

The media tested were: (1) complete transport medium, (2) the complete medium with the charcoal removed, (3) complete medium with the agar removed, (4) complete medium with the charcoal and agar removed. One milliliter of liquid fecal sample was mixed respectively with 2 ml of each of the transport media being tested. At designated intervals, a set of media was centrifuged at 850 xg and the supernatant fluids frozen at -20 C until observed by IEM.

In order to test for viral survival in diluted fecal fluids, a supernatant fluid was prepared from a fecal specimen obtained from a child infected with reolike virus. The fluid was placed into 2 tubes; one tube was incubated at 4 C and the other at 20 C. The tubes were sampled and observed periodically by electron microscopy for the presence of the virus.

A sample of fecal virus was filtered through a Millipore filter of 0.45 μm pore size. Antiviral antibody was incubated with the filtered virus preparation and a viral sample that had not been filtered. The two preparations were then examined by electron microscopy to determine the effect of filtration on the number of aggregates formed.

Specificity

Heterologous and homologous pairings of NCDV, reovirus and PI-3 virus with NCDV and reovirus antisera were examined by the IEM procedure of Vassal and Ray (1974). Mycoplasma arthritidis antiserum was paired with NCDV and reovirus. The samples were observed for the presence of aggregates.

In testing for auto-aggregation of the human reolike virus, a high titered virus sample was put into two tubes. One sample was filtered through a 0.45 μm pore size membrane filter, the other was not filtered. Each was again split and a tube with and one without anti-NCDV antibody were incubated, sampled and observed.

Two vials of antisera labeled Norden "A" and Norden "B" were received from Norden Laboratories. One contained anti-NCDV antibody and the other anti-coronavirus antibody but their identity was unknown. Dilutions of these antisera and a dilution of a known anti-NCDV antiserum were mixed with a suspension of NCDV. The samples were observed to find which of the unknown antisera had caused aggregation of the NCDV.

Viral Detection

Viral extracts were prepared from fecal samples obtained from scouring calves and from diarrheic infants. The method of Vassal and Ray (1974) was used to test for the presence of NCDV and the human reolike virus.

Using supernatant fluids from diarrheic calf and human feces, MDBK cells were infected and observed by the IFC technique of Barnett et al. (1975). These included samples that had been scored as positive and negative by IEM.

The FVPT of Foster, Peterson and Spendlove (1975) was used to test fecal fluids for the presence of NCDV. IEM positive and negative samples were used in performing the test.

Antibody Detection

Bovine and human sera were diluted and mixed with a NCDV or human reolike virus suspension. The Vassal and Ray (1974) method was used to detect the viral aggregates. The titers of the antisera were reported as the reciprocal of the highest dilution of antisera able to aggregate the virus.

RESULTS

Comparison of IEM Methods

The IEM methods of Vassal and Ray (1974); Kelen, Hathaway, and McLeod (1971); and Kapikian, Almeida, and Stott (1972) were compared using NCDV and the human reolike virus. The Kelen, Hathaway, and McLeod method required a fewer number of steps for the same end result than the method of Kapikian, Almeida and Stott. Vassal and Ray's method required a shorter incubation time with no high speed centrifugation or filtration step necessary and was more reliable than the other two methods tested (Table 1).

Table 1. Comparison of three IEM techniques in determining rapidity and sensitivity in NCDV detection

	Methods*		
Conditions	Kapikian	Kelen	Vassal
Incubation required for aggregate formation	20C for 1 hr 4C overnight	37C for 2 hr	37C for 1 hr
Centrifugation	17,300 xg for 1 hr	Not required	Not required
Filtration of Sample	Required	Not required	Not required
No. positive in two tests	1	1	2

*Kapikian, Almeida, Stott (1972)
Kelen, Hathaway, McLeon (1971)
Vassal and Ray (1974)

Conditions for Optimal Test Results

The data in Figure 1 show the effect of incubation time in NCDV detection using the method of Vassal and Ray (1974). More viruses and the greater number of viral aggregates were observed after 1 hour of incubation. The number of particles and aggregates rose and then declined sharply on either side of the optimal time. All subsequent tests used an incubation time of 1 hour.

The optimal temperature of incubation is 37 C as shown in Figure 2. The number of aggregates per grid square did not vary significantly, but the size of the average 37 C aggregate was 2.7 times larger than the next largest average aggregate at 4 C. The total number of particles present in the 37 C aggregates was 3.4 times the total number of particles present in the next largest, or 4 C aggregates. The 37 C incubation temperature was used throughout this study.

A test was performed to see if various agitating speeds during incubation of the virus-antibody mixture would affect the aggregate size and/or number. Table 2 shows that aggregate size and/or number is not influenced by varying the rotational speed of a shaker from 0 to 120 rpm during incubation. Excessive splashing would have resulted had the speed been higher.

The optimal negative staining time for the virus samples used in this study was found to be 30 seconds using a 2% PTA stain and the "grid on drop" technique previously described. Table 3 demonstrates the results of an average of several experiments. The 0.5% uranyl acetate at 30 and 60 seconds, and the 2% PTA at 60 seconds stained too heavily to be of any use in observing viral aggregates.

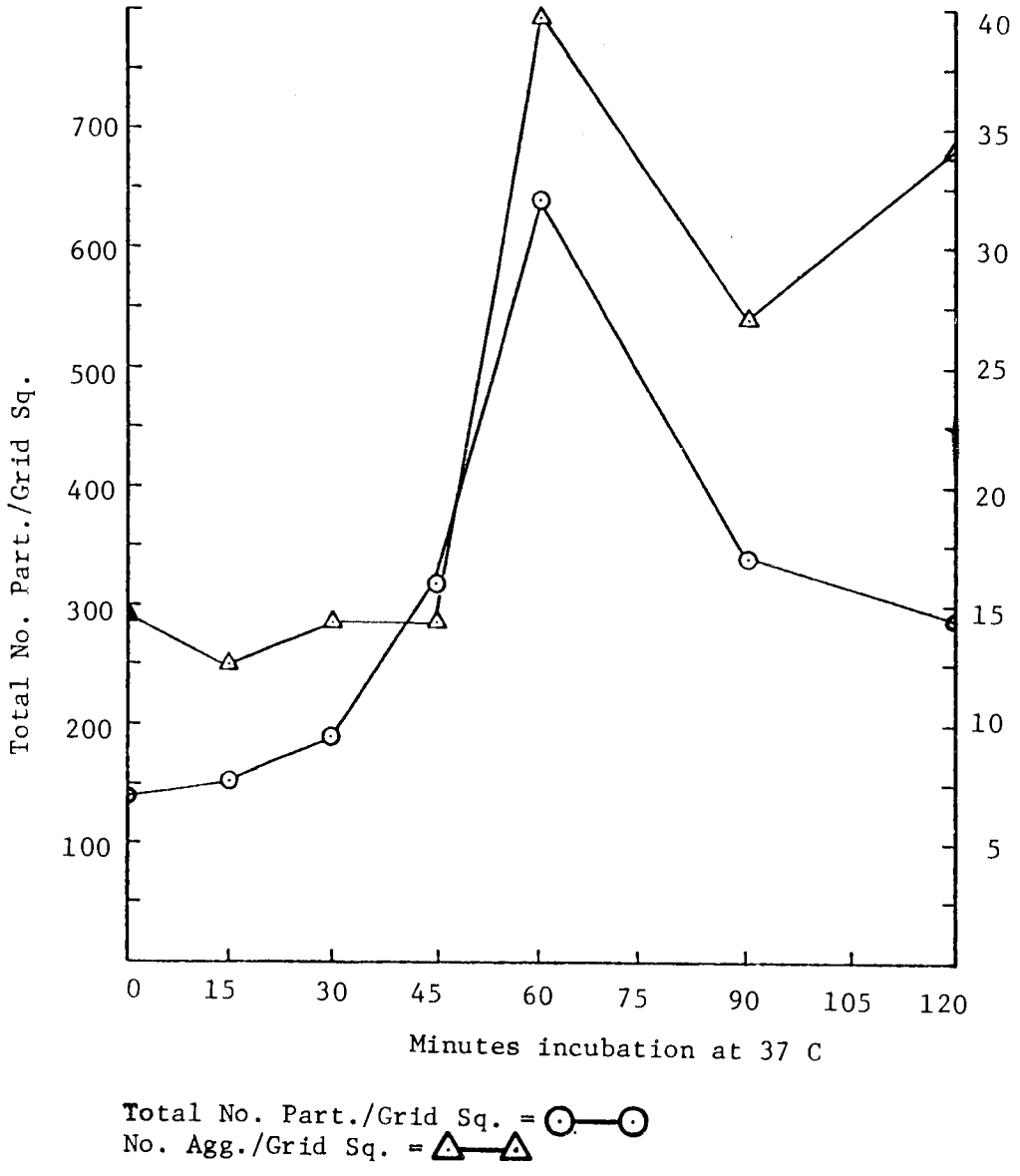

Figure 1. Optimal IEM incubation time. A comparison of the number of NCDV aggregates per grid square (No. Agg./ Grid Sq.), and the total number of particles per grid square (Total No. Part./Grid Sq.) from viral aggregates at various time intervals.

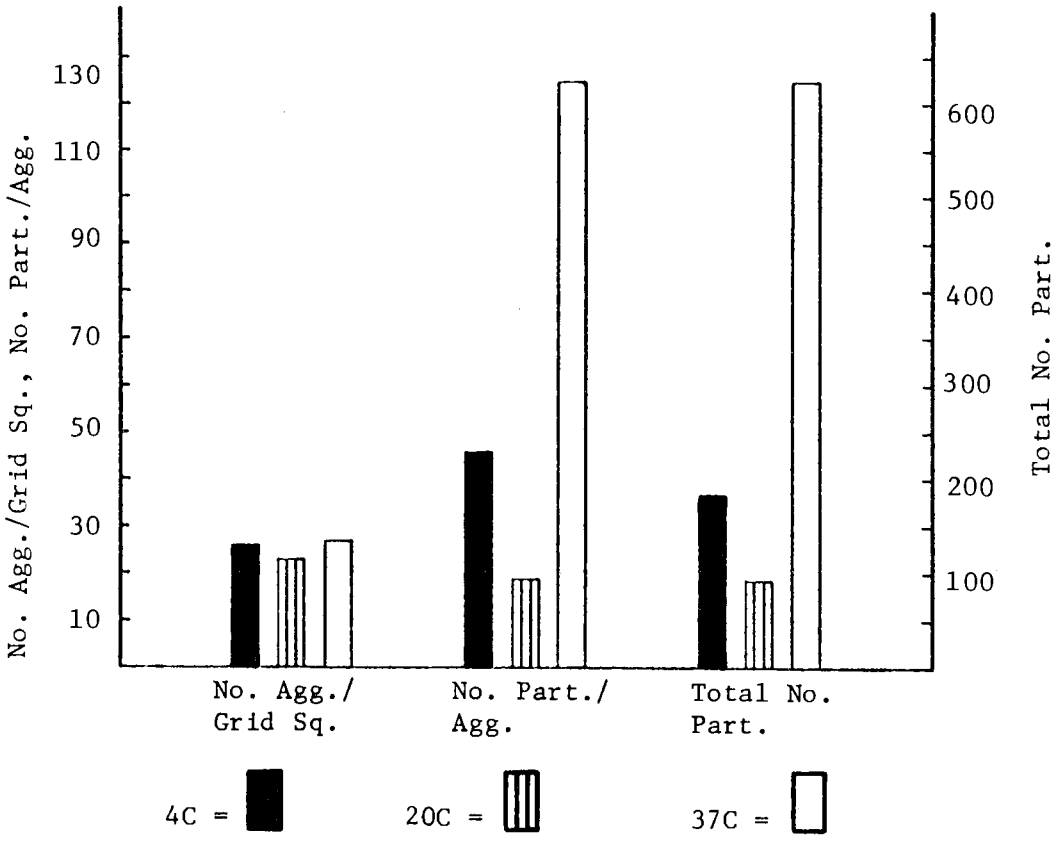

Figure 2. Optimal IEM incubation temperature. Comparison of the number of aggregates per grid square (No. Agg./Grid Sq.), the number of viral particles per aggregate (No. Part./Agg.), and the total number of particles (Total No. Part.) from viral aggregates at 4C, 20C, and 37C.

Table 2. NCDV aggregate formation during incubation at selected speeds in a shaker water bath

	RPM			
	120	110	95	0
No. aggregates/ grid square	6.3	3.8	3.3	6.7
Average no. particles/ aggregate	23	24	50	28
Total no. particles in aggregates counted	293	244	348	421

Table 3. Optimal negative staining of NCDV aggregates

Stain*	Method: Drop on grid	Method: Grid on drop	Stain time (sec.)	Results
PTA	X		30	Formvar layer broken
PTA		X	30	Good negative staining
PTA		X	60	Aggregates obscured by stain
UrAc	X		30	Aggregates obscured by stain
UrAc		X	30	Aggregates obscured by stain
UrAc		X	60	Aggregates obscured by stain

*PTA: 2% phosphotungstic acid
UrAc: 0.5% uranyl acetate

In preliminary experiments, it appeared that the Leibovitz (1969) viral transport medium, and modifications of it, improved viral detection from stool material. The transport medium, and transport medium with agar but without charcoal, seemed to yield the greatest number of aggregates per grid square. The agar held back much of the debris upon centrifugation resulting in a clear supernatant fluid.

The human reolike virus in diluted fecal fluids without transport medium was stable for at least 7 days at 4 C and 20 C after which the viral titer gradually declined over the next 14 days (Table 4). No virus was detectable at the end of 21 days in the 20 C tube while very few aggregates were present in the 4 C tube. Bacterial growth was present in both tubes after 14 days; this may have shortened the viral survival time.

The results in Table 5 demonstrate the effect that filtering has on the number of viral aggregates observable in a fecal sample. Filtering a sample through a membrane filter with a 0.45 μm pore size will usually reduce the number of aggregates. The reduction varied from 0 to 75% with an average of 18% fewer aggregates in the filtered sample.

Table 4. Effect of storage temperature on NCDV aggregate formation

	Relative no. of aggregates at:	
Day	4 C	20 C
0	+ + + +	+ + + +
7	+ + + +	+ + +
13	+ +	+
20	+	-

Table 5. Effect of 0.45 μm pore size filtration on NCDV aggregate detection in fecal supernatant fluids

Fecal sample	Unfiltered*	Filtered*	% Change
1	24	11	-54
2	11.5	20	+43
3	7	13.5	+48
4	6	1.5	-75
5	20.5	13	-37
6	6	6	0
7	156	52.5	-66
8	19	19	0
Average change			-18

*Numbers represent average number of aggregates per grid square

Specificity of IEM

NCDV is specific in its homologous and heterologous reactions with various antisera. As demonstrated in Table 6, viral aggregates formed in the presence of homologous antibody and were absent in heterologous parings. No discernable particles were observed in the PI-3 virus preparations. The presence of the viral envelope probably was a factor in rendering the PI-3 virus difficult to observe.

Auto-aggregation of reoviruses has been observed in high titered solutions (Spendlove, personal communication, 1974). No auto-aggregation of NCDV was observed with the fecal samples tested (Table 7). If the sample contained anti-NCDV antibody, aggregation occurred, but

Table 6. Specificity of IEM

Virus	Antiserum	Aggregates
NCDV	NCDV	present
NCDV	without	absent
NCDV	reovirus	absent
NCDV	*M. arthritidis**	absent
reovirus	reovirus	present
reovirus	without	absent
reovirus	NCDV	absent
reovirus	*M. arthritidis*	absent
PI-3**	without	absent
PI-3	NCDV	absent

* *Mycoplasma arthritidis*
** parainfluenza virus type 3

Table 7. Demonstration of lack of auto-aggregation in filtered (0.45 µm pore size) and unfiltered fecal supernatant fluids

Virus sample	Antiserum	Aggregates
filtered	present	present
filtered	absent	absent
not filtered	present	present
not filtered	absent	absent

if the filtered or unfiltered sample did not contain antibody, aggregation did not occur.

Upon incubation of a virus suspension with Norden antiserum "A" or Norden "B", it was found that NCDV aggregates formed in Norden "B" antiserum but none were found in Norden "A" antiserum. Table 8 shows the results of the experiment using tissue culture grown virus and virus extracted from stool material.

A letter sent under separate cover by Michael Gill of Norden Laboratories identified Norden "A" as coronavirus antiserum and Norden "B" as reovirus (NCDV) antiserum. This information was not received until after the test had been completed.

Table 8. Testing of coded antisera against NCDV positive fluids

NCDV in:	Antiserum	Aggregates
tissue culture fluid	Norden A*	absent
tissue culture fluid	Norden B**	present
tissue culture fluid	Rabbit anti-NCDV	present
fecal supernatant fluid	Norden A	absent
fecal supernatant fluid	Norden B	present
fecal supernatnat fluid	Rabbit anti-NCDV	present

*Anti-coronavirus antiserum

**Anti-NCDV antiserum

Viral Detection

A total of 43 fecal samples from diarrheic calves were tested for the presence of NCDV. Of the 43 samples, 35 different animals were represented. Two of the 8 remaining fecal samples were collected from 2 previously sampled animals while the remaining 6 specimens were obtained from the gastrointestinal tracts of animals after death. Of the 35 specimens, one had a Salmonella typhimurium infection. Of the reamining 34 samples, 14 or 41% were NCDV positive. Ages of the calves ranged from 1 to 21 days; all of the NCDV positive calves were from 1 to 6 days old.

In one herd of 6 calves, all 6 became infected with NCDV, and 3 died. Virus was detectable 6 days but not 8 days after clinical symptoms appeared in 2 of the animals tested. Upon examination of calf gastrointestinal tracts, NCDV aggregates were found in 2/2 of the colon and rectum samples, 1/2 of the ceacum samples and 0/2 of the small intestine samples.

Ten of the 14 IEM positive calf specimens were negative when examined for infectivity. None of the negative IEM samples were positive by the IFC test. In the positive IFC test samples, infectious titers ranged from 1.6×10^4 to 3.2×10^5 infectious particles/ml of supernatant fluid. Some specimens were toxic to the cells even though each had been used undiluted and diluted 1:4 or 1:8. Cells in some cultures were killed while others appeared normal after inoculation and overnight incubation of the sample; consequently, some tests may have been negative as a result of toxic materials in the stools.

In all tests scored as positive, viral aggregates were observed in the first of three grid squares routinely examined with the exception of two samples. In these samples, aggregates were found in the second grid square.

The positive samples were from herds in Amalga, Benson, Newton, Richmond and Smithfield, Utah.

Five children were tested for the presence of human reolike virus with a total of 9 samples collected. Of the 5 children, 4 or 80% were positive for the virus. The age range of the children was from 11 to 24 months. The negative sample was in a 12 month old child. Virus was found from the second to seventh day after clinical symptoms appeared. A sample was not obtained on day one and the virus was not detected in the day eight sample. The fecal material in the positive samples was a white-yellow, semi-solid to pasty material. Four children in Logan, and 1 child in Collingston, Utah were found positive for the human reolike virus.

No sample or any dilution thereof of any of the IEM tested human fecal samples was found to be infective in the IFC assay. Cells in some of the cell monolayers were killed while others appeared healthy.

When the IEM nad FVPT methods were used for detecting NCDV in fecal specimens, a 100% correlation of results was observed. Of the 29 calf samples tested by the FVPT, 12 contained fluorescent aggregates. These same 12 samples were positive when observed by IEM.

Antibody Detection

Of the 16 calf and adult bovine serum samples tested, 16 or 100% contained antibody against NCDV. Three of these were from NCDV

infected calves. The calf antibody titers ranged from 1:32 to 1:256. The adult serum samples ranged in titer from 1:4 to 1:256. The calf sera samples were from animals in Smithfield, Utah. The adult bovine sera were from herds in Shelley and Preston, Idaho, and Parowan, Richmond and Wellsville, Utah.

The human infant serum sample tested had a titer of 1:2048 against the human reolike virus.

The photomicrographs in Figure 3 demonstrate the NCDV aggregates formed with varying amounts of anti-NCDV antiserum. Three viral particles constitute an aggregate; an example of a 3-virus aggregate is shown in Figure 4. Figure 5 is a low power electron micrograph of human reolike virus aggregates to show abundance of immune complexes. The human reolike virus is shown in Figure 6. In Figure 6a, the virus is aggregated by anti-NCDV antiserum and in Figure 6b anti-human reolike convalescent serum has aggregated the virus particles.

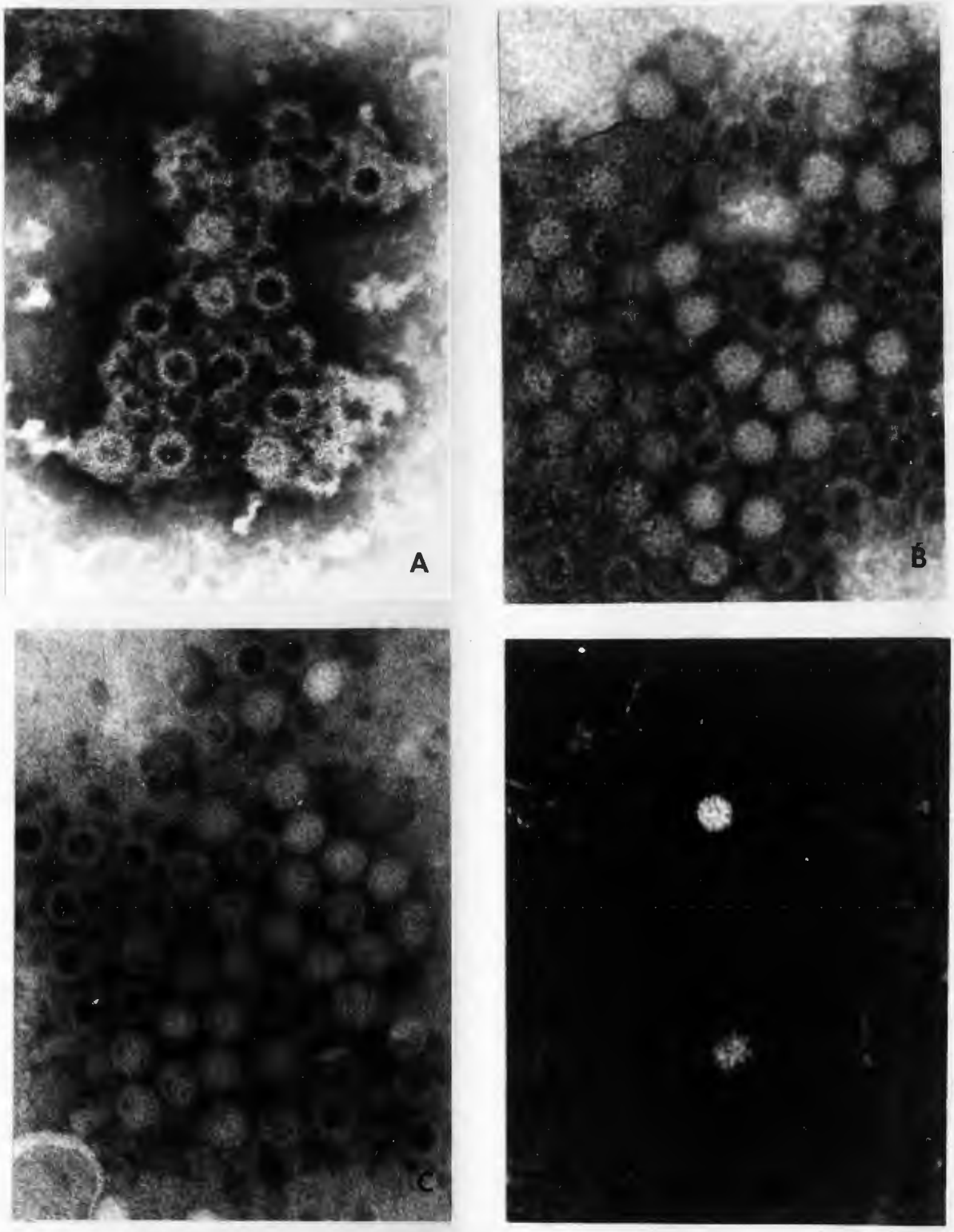

Figure 3. Electron micrographs of NCDV aggregates containing various dilutions of antibody: (a) 1:32, (b) 1:128, (c) 1:512, and (d) no antibody. (115,500 X)

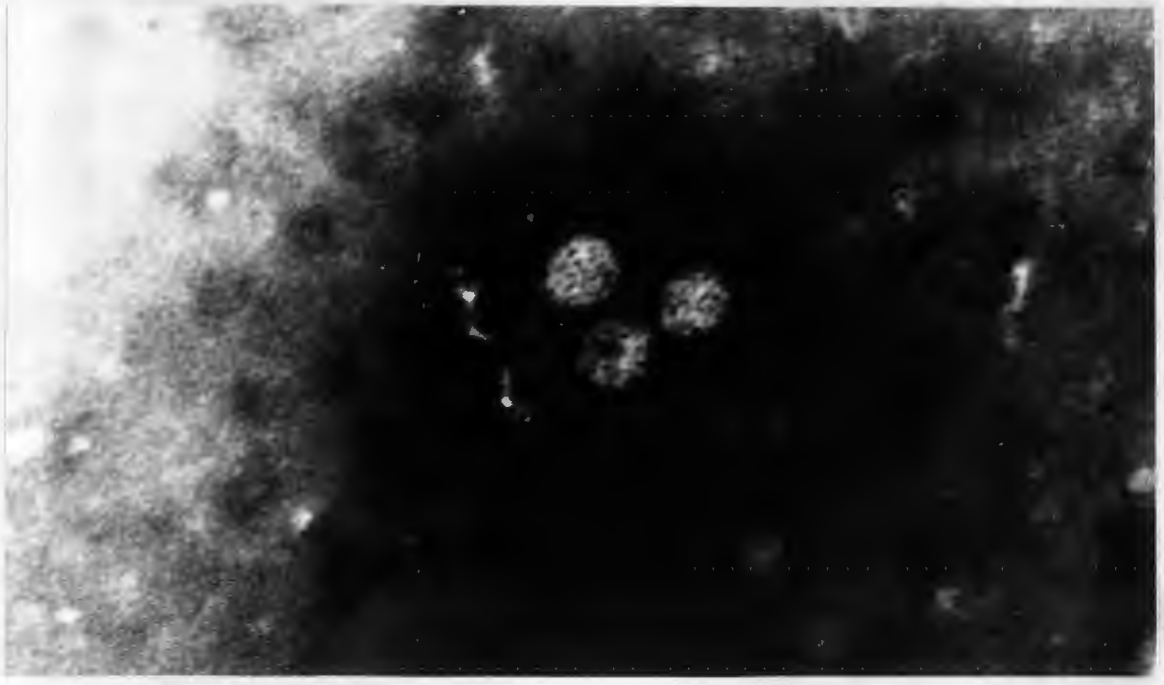

Figure 4. A minimal size aggregate of NCDV. (165,500 X)

Figure 5. A low power micrograph of human reolike virus aggregates demonstrating abundance of immune complexes. (33,000 X)

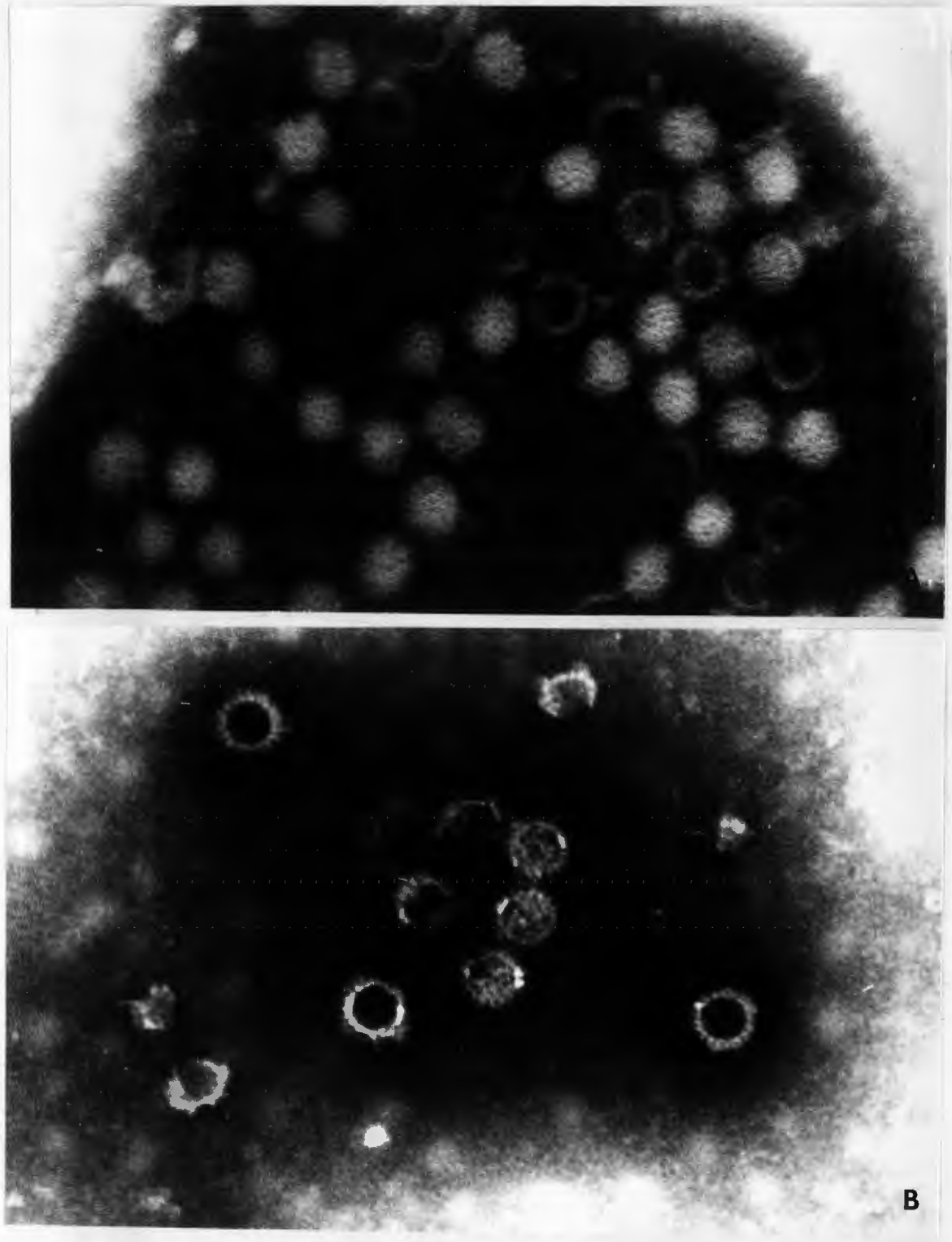

Figure 6. Electron micrographs of human reolike viruses aggregated by (a) anti-NCDV antiserum and (b) anti-human reolike convalescent serum. (165,500 X)

DISCUSSION

The primary purpose of this study was to detect the presence of neonatal calf diarrhea virus (NCDV) and the serologically related human reolike virus in northern Utah. A rapid and sensitive method was needed to expedite the testing of many samples. The immune electron microscopic method (IEM) and fluorescent viral precipitin test (FVPT) were the tests adapted and used in this study.

Prior to developing of the IEM and FVPT methods, the diagnostic methods of choice were the electron microscopic (EM) examination of purified fecal supernatant fluids (Turner, Caple and Craven, 1973), or the use of coverslip cultures in the immunofluorescent cell (IFC) assay for NCDV (Barnett et al., 1975). The EM method provided good morphological data on the virus and the IFC assay made it possible to obtain valuable information about the growth characteristics of NCDV. Drawbacks to these methods for diagnostic work were the extensive concentration and purification needed for EM and the inability to detect total particles in the IFC assay.

The IEM procedure has been a valuable tool in viral detection. Antiserum aggregates the virus, so observation can be made after limited purification. This has made IEM sensitive and rapid. With the establishment of optimal reaction, staining, and specificity conditions, along with some outlines for viral survival times, the IEM procedure enables the observation of NCDV in fecal supernatant fluids.

The aggregated, stained virus can be kept for at least 3 months. The proteins or other material in the crude extract offers a fixative quality that preserves the aggregates. This test might be improved as a diagnostic test if more information were obtained on viral and antisera storage, and also on ways to increase sensitivity, such as short, low speed centrifugation of the aggregated sample.

The FVPT was developed late in the study, but its usefulness was apparent when 100% correlation occurred when tested against the IEM method. The sensitivity of the FVPT is dependent in part on the viral aggregate size in the suspending medium. Aggregates with 3 NCDV particles on a side could be resolved at the 1250X magnification that is used in the FVPT. With viral aggregates of 25 and more particles a common occurrence, and with a much larger sample size ($\sim$0.05 ml in the FVPT compared to the amount of sample adhering to an electron microscope grid), the FVPT can be used as a sensitive diagnostic tool. Although the aggregate numbers decrease upon filtration (Table 5), this decrease is compensated for by using a larger sample size.

Another use of the FVPT is the specific reaction with anti-viral antibody. Globulin preparations that are overlabelled with fluorescein can be used because centrifugation and filtration remove the microscopically visible debris that would be stained. The indirect method of fluorescent antibody staining can be adapted to the FVPT. This is advantageous to the diagnostic laboratory because maintaining a large stock of conjugates is not necessary in detecting a variety of viral agents.

Because of its sensitivity, specificity, and use of relatively inexpensive and maintenance free instruments, the FVPT may become a valuable diagnostic tool in the future. Further studies are needed on agents detectable by the FVPT, application of the method in antibody level determinations, detection of viral particles in purification procedures, and rapid identification of viruses in communicable diseases.

The survey for NCDV indicated that it is infecting calves in northern Utah and its 41% incidence rate in diarrheic calves makes it an agent of major importance. The virus was found throughout northern Cache Valley indicating its widespread distribution in this area.

Within 72 hours after placing the 6 calves together that were purchased at an auction from various herds, all had NCDV induced diarrhea. The 6 calves were housed together in a barn and all other animals tested were kept in individual pens. The rapid spread and high incidence of infection may be partially due to the confined living conditions.

The importance of a total particle, viral diagnostic procedure was demonstrated when IEM, FVPT, and IFC methods were compared. Not only are the IEM and FVPT methods more rapid, but viral particles that may have been inactivated due to sampling or storage methods are still detectable.

In detecting an infectious agent, proper samples must be taken. This was well demonstrated when viral particles were detected in the lower gastrointestinal tract, but were absent in samples obtained from the small intestine of infected calves.

The widespread nature of NCDV was demonstrated by the high incidence of anti-NCDV antibody. The antibody level is probably maintained by repeated exposures of animals that have had an initial sub-clinical or clinical infection. More studies need to be conducted to determine antibody levels in bovine age groups and to determine the effect of exposure to NCDV on the antibody level. Investigations such as these may provide a correlation between the level of antibody in the herd and the possibility of an NCDV outbreak.

When discussing with local physicians the possibility of conducting a survey on viral caused infant diarrhea, two main statements were made. First, an etiological agent for the vast majority of human infant diarrhea cases has not been identified; consequently, a non-bacterial cause is indicated. Second, the majority of cases occur in the warmer months. This explains the scarcity of samples available in this study.

Two of the four positive human fecal samples were obtained from infants of students working with NCDV. A close serologic relationship between NCDV and the human reolike virus has been established (Flewett et al., 1974). This observation was confirmed by the aggregation of the human virus with anit-NCDV antiserum as well as with infant convalescent serum. Because of the close serologic relationship of these agents, the IFC assay was implemented to determine if detection of infectious human reolike virus was possible in MDBK cells. The lack of fluorescence in the cells exposed to stain indicated that no growth occurred. If growth had occurred, the fluorescein labeled

anti-NCDV conjugate probably would have stained the viral particles. The reason being that fluorescent aggregates were observed in the FVPT when fluorescein labeled anti-NCDV conjugate was combined with the human reolike virus. Further studies should be conducted to determine the incidence, spread, and in vitro growth of the human reolike virus.

SUMMARY

The purpose of this study was to detect neonatal calf diarrhea virus (NCDV) and a related human infant reolike virus from fecal specimens collected in northern Utah. Using immune electron microscopy (IEM) and to a limited extent, the fluorescent viral precipitin test (FVPT), a survey was completed.

Using an adaptation of an IEM procedure, a rapid, specific, and sensitive test was developed. Viral aggregates formed when crude fecal supernatant fluids and anti-NCDV antiserum were mixed. Optimal IEM conditions for use with NCDV were developed. Results indicated the incubation time and temperature, and method of sample preparation and storage affected the reliability of the IEM results.

The FVPT results had a perfect correlation with results obtained in the IEM method. Because it is rapid, can be adapted to the indirect test, and can detect viral aggregates with dimensions of 0.2 μm, the FVPT is a valuable and versatile tool that should find general acceptance and application.

The results have shown that NCDV and the human infant reolike virus, and their respective antibodies, are present in the bovine and human populations of this geographical area.

The IEM and FVPT methods for NCDV and human infant reolike viral detection are superior to other methods currently in use because of their rapidity, specificity and sensitivity due in part to their ability to detect viable and inactive viruses.

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