THE ANTIGENIC RELATIONSHIPS AMONG BOVINE VIRAL DIARRHEA VIRUS ISOLATES

by

BASHIR HAMID TEIRAB

B.V.Sc., University of Khartoum, Sudan, 1966

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Infectious Diseases

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1977

Approved by:

Harold C. Minocha

Major Professor

THIS BOOK
CONTAINS
NUMEROUS PAGES
WITH THE ORIGINAL
PRINTING BEING
SKEWED
DIFFERENTLY FROM
THE TOP OF THE
PAGE TO THE
BOTTOM.

THIS IS AS RECEIVED
FROM THE
CUSTOMER.

Document
LD
2668
T4
1977
T45
C.2

DEDICATION

To my parents for their love and to the people and Government of Sudan for their support.

TABLE OF CONTENTS

	Acknowledgements	iv
	Abbreviations	v
I.	Introduction	1
II.	Literature Review	4
III.	Materials and Methods	17
	BVD Viruses Used	18
	Cell Cultures and Media	20
	BVD Virus Assay	21
	BVD Virus Concentration and Purification	22
	Neutralization Tests	25
IV.	Results	27
	BVD Virus Plaque Characteristics	28
	BVD Virus Concentration and Purification	28
	Serum Neutralization (SN) Tests	30
V.	Tables and Figures	35
VI.	Discussion	52
	BVD Virus Plaque Characteristics	54
	BVD Virus Concentration and Purification	55
	Serum Neutralization Tests	61
VII.	Appendix	65
	Materials and Methods	66
	Results	67
	Discussion	68
	Tables and Figures	71

VIII. Reagents and Suppliers' Addresses 79

IX. References 84

ACKNOWLEDGEMENTS

The author would like to express his sincere appreciation to Dr. H. C. Minocha, major professor, for his constant encouragement and guidance during the course of this investigation and for his advice in the preparation of this manuscript. The author would also like to thank the committee members, Dr. R. A. Consigli and Dr. A. L. Burroughs for their suggestions and friendship during the research. Thanks are due to Dr. E. H. Coles for providing laboratory facilities in the Department of Infectious Diseases. The statistical analyses contributed by Dr. C. Loschcke were invaluable and gratefully acknowledged. I would like to express my great appreciation to Mrs. Eva Graybeal for her technical assistance in the laboratory.

ABBREVIATIONS

ATCC	American Type Culture Collection
BVD	Bovine Viral Diarrhea
^{o}C	Degree Centigrade
CC	Cubic Centimeter
CPE	Cytopathic Effect
DNase	Deoxyribonuclease
Dil	Dilution
EMB	Embryonic bovine kidney
g	Gravity
IBR	Infectious bovine Rhinotracheitis
MDBK	Madin Darby Bovine Kidney
MEM	Minimal Essential Media (Eagle)
mg	Milligram
ml.	Milliliter
M_1-M_4	BVD Minnesota field isolates
NADL	National Animal Disease Laboratory
NaPTA	Sodium Phosphotungstate
NCP	Non Cytopathogenic
NDV	Newcastle disease Virus
PBS	Phosphate Buffered Saline
PFU	Plaque forming units
PI_3	Parainfluenza 3
PSK	Penicillin-Streptomycin-Kanamycin

RNA	Ribonucleic Acid
RNase	Ribonuclease
SN	Serum Neutralization
TEN buffer	Tris-EDTA-NaCl
µg	Microgram
VD	Virus Diarrhea
V/V	Volume per Volume
W/V	Weight per Volume

SECTION I

INTRODUCTION

INTRODUCTION

Bovine viral diarrhea (BVD) virus has been implicated in bovine respiratory disease complex. The economic losses due to the disease are extensive among cattle populations. Many etiological agents have been shown to be responsible for respiratory disease in cattle, e.g. bovine viral diarrhea (BVD) virus, infectious bovine rhinotracheitis (IBR) virus, parainfluenza 3 (PI_3), bovine rhinovirus, adenovirus, reoviruses, mycoplasma, and pasteurella (Bruner and Gillespie, 1973; Thomson, 1974; Phillip, 1968). The principle viral agents responsible for the syndrome are BVD, IBR and PI_3. Circulating antibody and/or hemagglutination titers to all three viruses have been detected concurrently in the same animal (Jensen and Mackey, 1971).

Commercial vaccines against BVD consisting of attenuated viruses grown in tissue culture (Bittle, 1968; and Coggins et al., 1961) are somewhat accompanied by hazards of post vaccinal reactions in cattle (Clark, 1968; McKercher et al., 1968; Peacock, 1968; Peter et al., 1967; and Rosner, 1968) and have been suspected not to protect all vaccinated animals. This may possibly be due to different types of virus strains used for vaccine production.

The antigenic relationships among different isolates of BVD viruses have been studied by many investigators (Fernelius et al., 1971a; Castrucci et al., 1968a, b; and Castrucci

et al., 1975). Significant serological differences among BVD virus isolates as revealed by neutralization indices are not adequate for their classification into distinct serological groups (Corthier and Aynaud, 1973). This classification would require a detailed comparative study of the BVD virus isolates by cross serological reactions in order to explore the extent of antigenic relationships among various strains.

Animals inoculated with BVD virus isolates serologically unrelated on the basis of cross neutralization reactions, demonstrated cross protection in animals challenged 38 days after vaccination (Castrucci et al., 1975). However, cross protection experiments were not carried out for longer periods to test the duration of immunity.

The objective of this study was to: (1) Propagate, concentrate and purify three prototype strains and four field strains of bovine viral diarrhea (BVD) virus and (2) Establish the antigenic relationships among these viruses by reciprocal cross serum neutralization tests.

SECTION II

LITERATURE REVIEW

LITERATURE REVIEW

History of Bovine Viral Diarrhea (BVD) Virus: Bovine virus diarrhea was first reported as a transmissible disease of cattle by Olafson et al., 1946 in New York; as an X disease of cattle in Canada by Childs in the same year, and as an epizootic enteritis occurring in Sweden by Hedstrom et al., 1951. In 1953 it was reported as a mucosal disease by Ramsey and Chivers in the Midwest. Baker et al., 1954, recovered two strains of virus diarrhea (VD) virus from cattle and reproduced the disease in calves. Wheat et al., 1954, isolated a VD strain from cattle in California that was found to be immunologically identical with a New York strain. Since then the disease has been reported in most parts of the U.S. and several other countries in the world.

Epidemiology and Symptomatology: Bovine viral diarrhea virus has been incriminated in a variety of cattle diseases causing respiratory illness, diarrhea, abortion and lesions of mucosa of the alimentary canal (Kahrs, 1971, Kahrs et al., 1970a, Gillespie et al., 1967, Kahrs, 1968 and Kahrs et al., 1970b). Bovine viral diarrhea is not related to rinderpest, IBR and parainfluenza-3 (Howarth, 1972, Walker et al., 1947), but there is a minor antigenic relationship between BVD virus and hog cholera virus (Darbyshire, 1962, Shiffy et al., 1962, Mengeling et al., 1963). The subclinical infection of BVD virus has become so widespread in the United States that

50-60% of all cattle tested have antibodies (Pritchard, 1963, and Howarth, 1972). The respiratory spread is the most important mode of transmission of BVD virus but it may be transmitted via urine (Pritchard, 1963, Howarth, 1972).

Isolation of Bovine Viral Diarrhea Virus: Underdahl et al., 1957, reported the isolation of two cytopathic agents but they failed to identify the virus isolates as the causative agents of the disease. Gillespie et al., 1960, identified a cytopathic virus in Oregon. This strain C24V has become one of the viral prototypes in laboratories throughout the world. Burki in 1965 investigated the quantitative distribution of the virus in calves with naturally occurring infections and concluded that the greatest amount of the virus was in the mucous membrane of the alimentary canal. In subclinical infection the virus was isolated from washed leukocytes even when serum antibody was present (Gutekunst et al., 1964). Gratzek in 1962 compared two strains of virus, C24V and a non-cytopathic New York (N.Y.) strain of bovine virus diarrhea on the basis of the plaque production, growth curve, sensitivity to ether, cytopathic effects in cell cultures, interference test, and adsorption. He concluded that virus-infected cells were able to undergo mitosis and form clones. Using primary cell cultures of embryonic bovine kidney, Gillespie et al., 1963, reported the greatest yield of infective virus 72 to 96 hours after inoculation; the concentration of infective free virus

was greater than the cell associated virus. Mills et al., 1965, found that cell cultures of the kidneys of young calves were more practicable for C24V propagation than embryonic cell cultures. BVD virus strain NADL was adapted to swine kidney cell culture by Malquist et al., 1965; early infection by hog cholera (HC) virus abolished the effects and yield of the adapted BVD virus. Fernelius et al., 1966, recommended the use of specific pathogen-free animals for viral passages, antiserum production and cell culture systems. NADL strain of BVD virus was adapted to Madin-Darby bovine kidney (MDBK) cells after first carrying it through six passages in bovine testicle cell cultures (Marcus et al., 1968). Fernelius et al., 1969a, using fluorescent antibody (FA) technique, were able to demonstrate abortive infection in hamster kidney and in human cells. The cytopathic changes in cells, particularly bovine embryo kidney and bovine embryo testicle as reported by Singh, 1969, include elongation and shrinking of cells and varying degrees of vacuolation of the cytoplasm. Fernelius et al., 1969b, demonstrated that the expression of cytopathogenicity of NADL and cell-line adapted NADL viruses in embryonic bovine kidney changed during rabbit passages to resemble cytopathogenicity of Oregon-C24V strain of BVD virus (biotypic conversion). Cancellotti et al., 1972, were able to isolate a cytopathogenic BVD agent on spleen and bovine fetal kidney cells and they found that fetal spleen cells

were more sensitive to the cytopathogenic action of the virus than did bovine fetal kidney cells. BVD virus concentration increased 150,000 fold by day 4 with as much as 96% of the inoculum adsorbed by macrophages grown in suspension (Truitt et al., 1973). It was later found that bovine fetal spleen cells adsorbed BVD virus faster than bovine fetal kidney cells (Schiff et al., 1973).

Classification of BVD Virus: The morphology of BVD virus has not been clearly elucidated (Andrews Pereira, 1972). Hermodsson and Dinter 1962 described particles with a diameter of 40 nm., but were unable to confirm that the particles were BVD virus. Pritchard, 1963, using chromium shadowing revealed particles of 35 to 55 nm. in diameter. Ditchfield and Doane, 1964, have described BVD virion as 150 to 250 nm., in size with a structure similar to that of a myxovirus. Dutta et al., 1964, have described an intermediate size of 80 nm. Hafez et al., 1968, believed that the size of BVD virion varies between 22 and 90 nm. Ritchie and Fernelius, 1969, suggested a size of 80 to 100 nm. particles. Maess and Reczko, 1970; Horzinek, et al., 1971, have suggested particle size of 57 nm. and an inner core of 24 nm. in diameter. Castrucci, 1968a, b, described two BVD virus isolates which were shown to be RNA containing lipovirus, sensitive to pH3, 57-58 nm. in diameter and lacking hemagglutination and hemadsorption properties. Scott et al., 1974, have found pleomorphic but roughly spherical

particles of 50 to 80 nm. in diameter. These particles resemble rubella virus but they display a mottled appearance inside the outline of the outer membrane. These workers supported the suggestion of Horzinek, Maess and Lauf, 1971, that BVD virus should be classified as a togavirus (lipid containing RNA virus with cubic symmetry of the nucleoeapsid) although BVD and hog cholera viruses differ from the other togaviruses in their lower sedimentation and density values, their nucleoeapsid diameters and the unusual "rosary" envelope structure (Horzinek et al., 1971). Gillette, 1966, Tanaka et al., 1968, and Hafez et al., 1972b, using DNA inhibitors have reported that BVD viral core contains RNA. Further evidence of RNA contents in BVD viruses was obtained by nucleic acid extraction (Diderholm and Dinter, 1966, Moennig, 1971).

Experimental Reproduction of BVD Virus in Cattle and Other Animals: Bovine viral diarrhea can be reproduced experimentally (Olafson et al., 1946, Baker et al., 1954, Pritchard et al., 1955, Carlson et al., 1957). Experimentally induced BVD in cattle is often mild but may be severe and fatal. Bovine viral diarrhea virus was propagated in cattle, sheep, and goats, but not in pigs, cats, mice, suckling mice, hamster, ferrets, pigeons, day-old chicks and guinea pigs (Pritchard 1963). The highest viral titer of the blood and spleen of calves occur four or five days postinoculation. Attempts to cultivate BVD virus in embryonated chicken eggs had not been successful

(Olafson and Richard, 1947; Baker et al., 1954; Pritchard et al., 1956; and Huck, 1957). Bovine viral diarrhea virus can be propagated in cell cultures and some isolates, e.g. New York I is noncytopathogenic while others produce cytopathic effects in numerous cell culture systems. Lee and Gillespie (1957) cultivated BVD New York I virus in cultures of bovine skin and muscle tissue and bovine embryonic kidney cells.

Naturally occurring antibodies to BVD virus were first found in Australian pigs (Snowdon, 1968). Naturally occurring infection of sheep with BVD virus has not been reported but experimental inoculation of ewes caused the birth of lambs with congenital malformation such as cerebellar hypoplasia (Siebold, 1967, George, 1971, and Ward, 1961). Lambert et al., 1969, observed the survival of calves following an in utero infection with BVD virus and suspected those calves might have developed an immune tolerance with persistent viremia. Such animals are slow gainers and do poorly. Ward et al., 1969 had shown that calves which had their dams injected with BVD virus had antibody titers at birth and the antibodies were maintained for six months. When bovine fetuses were injected with BVD virus at different gestation stages they gained immunologic competence to the virus and were shown to be capable of producing serum neutralizing antibodies by 200 day of gestation (Braun et al., 1973). Bovine viral diarrhea virus was recovered from the placentomes and fetuses of ewes 14 days after

inoculation and was also recovered from the fetal cotyledons and uterine caruncles at Lambing (Hore et al., 1973). Different strains of BVD virus when tested in ewes at various stages of gestation showed different degrees of pathogenicity causing abortion, fetal death, mumification and resorption or expulsion of the fetus (Snowdon, et al., 1975).

Purification of Bovine Viral Diarrhea Virus: The separation of BVD virus structural proteins by polyacrylamide gel after disruption by various techniques has been reported by Frost and Leiss (1973) and Pritchett and Zee (1975). Disruption of BVD virus preparation with 1% sodium dodecyl sulphate (SDS) resulted in the production of three electrophoretic components (Frost and Leiss, 1973). Purification of BVD virus by differential and isopycnic-zonal centrifugation proved ineffective, as residual host proteins contaminated the viral preparations (Pritchett and Zee, 1975). Coelectrophoresis of 3H-labeled virus and 14C-labeled host material did provide a way to distinguish between viral proteins and host proteins and four electrophoretic components of viral origin were identified and their molecular weights estimated. Although the structural proteins of BVD virus have not been unequivocally characterized, it is apparent that the number and the molecular weights of the proteins more closely resemble those of group A rather than group B togaviruses (Pritchett and Zee, 1975). BVD virus was purified partially by agar gel filtration (Fernelius and

Velicer, 1968) and by the use of sucrose, $CsCl_2$ and potassium tartrate density gradients (Maess et al., 1970), Horzinek et al., 1971, Fernelius 1968; Ritchie and Fernelius 1969, Hafez et al., 1972b, Parks et al., 1972). The buoyant density of four strains of BVD virus: NADL, C24V, CG-1220, and New York I grown in primary embryonic bovine cells was 1.15 g/cm^3 (Fernelius 1968) and the buoyant density of BVD virus strain C24V, propagated in calf testicle was 1.115 g/cm^3 (Maess et al., 1970). Parks et al., 1972, purified BVD virus strain NADL and recovered the infectious virus with density of 1.092 g/cm^3. Scott et al., 1974, had reported a 90% virus recovery in 15-30% sucrose gradient with buoyant density of 1.11 g/cm^3.

Serology of Bovine Viral Diarrhea Virus: Cross protection tests in cattle and tissue culture indicate that virus diarrhea and mucosal disease are caused by the same virus (Howarth, 1972). The existence of serologic relationship among the strains of BVD virus has been reported by many investigators (Darbyshire, 1963, Dinter, 1964, Gillespie et al., 1960, Gillespie et al., 1961, Gillette, 1966, Kniazeff and Pritchard, 1960, Kniazeff et al., 1961, Mills et al., 1965, Noice and Schipper, 1959, Taylor et al., 1963, Thomson et al., 1963, Castrucci et al., 1968a, b). Pritchard et al., 1956, failed to cross neutralize New York and Indiana strains of BVD virus.

Antigenic relationships between BVD virus strains have been observed (Gillespie and Baker, 1959, Gillespie et al.,

1960, Gillespie et al., 1961). Kniazeff et al., 1960, by plaque reduction test had neutralized C24V by antisera prepared against strains from New York, Nebraska, Indiana, Iowa, North Dakota, and England. The same antigenic relationship was also reported by Kniazeff et al., 1961, between BVD virus strains from West Germany, England, Scotland, and the United States, however the extent of the relationship was not determined.

In 1964 Gutekunst and Malquist compared the complement-fixing and serum-neutralizing antibodies against the cytopathogenic NADL and noncytopathogenic CG-1220 strains of BVD virus by reciprocal cross neutralization tests and observed no differences in complement fixing (CF) antibody whereas serum neutralizing antibody appeared to be strain specific. Fernelius in 1964 compared cytopathogenic and noncytopathogenic BVD viruses and showed the occurrence of common as well as strain specific antigens. Tyler et al., 1965, have shown a close relationship between the isolates of mucosal disease which included the Sanders, Merrel, NADL, North Dakota (BMD) and Nebraska (C-I). The comparison was done on the basis of the clinical, hematologic, immunologic and pathologic responses in calves. No conclusive evidence regarding the antigenic relationship was observed. In 1967 Chennekatu and coworkers have speculated about the possibility of an immune failure or an immune delay due to the destruction of lymphoid tissue and

an inhibition of the proliferation of surviving immunologically competent cells of the germinating center. These workers did not speculate about the antigenic diversity of the BVD virus disease complex. Castrucci et al., 1968a, have shown that the TVM_1 and TVM_2 BVD virus isolates are antigenically identical whereas three strains from the United States (C60F, NADL, and 65/150) differ from TVM strains.

Fernelius in 1968 has grouped BVD viruses in order of decreasing cytopathogenicity for susceptible host cells as NADL >C24V >CG-1220 >Indiana >Merrel >New York I >Sanders. Taylor et al., 1963, demonstrated a reciprocal immunologic relationship between several BVD-virus strains including C24V which was shown not to adapt to eggs, suckling mice, or rat fetuses but did adapt to lamb fetus in utero. Fernelius et al., 1971a, have proposed three serotypes for classifying BVD virus strains on the basis of cross neutralization test and concluded that these BVD virus strains are serologically identifiable. Cytopathogenic strains of BVD virus differ with regard to type and degree of the CPE (Fernelius and Ritchie, 1966, Hafez et al., 1972a) as well as to plaque size (Gratzek et al., 1967 and Hafez et al., 1972a). The presence of different plaque morphology was not controlled by the viral genome and might be due to differences in adsorption, penetration and cell response (Hafez et al., 1972a). Several investigators (Borgen, 1963, Taylor et al., 1963, Castrucci et al., 1968a,

and Hafez et al., 1972a) reported failure to demonstrate hemagglutination or hemadsorption by BVD virus strains. Corthier and Aynaud, 1973, observed some significant serological differences between C24V and NADL strains of BVD virus and were not able to classify them into distinct serological groups on the basis of neutralization indices. Castrucci et al., 1975, have indicated that wide serological differences among BVD viruses are not related to their immunizing properties and that vaccines prepared from different BVD viruses provide protection against other virus strains.

Vaccination of BVD Virus: The development of BVD virus vaccines was based on the attenuation of viral agents by serial passage in rabbits (Baker et al., 1958) or in bovine kidney cell culture (Coggins et al, 1961). Commercial vaccines consisting of cell cultures attenuated C24V strain (Bittle, 1968, Coggins et al., 1961) or NADL strain adapted to swine kidney cell line (PK-15) (Gutekunst, 1968) are usually multivalent (Baker et al., 1958, Bittle et al., 1968, and Tamoglia, 1968). It was observed that the use of live-virus vaccines was accompanied by post-vaccinal reactions (Clark, 1968, McKercher, 1968, Peacock, 1968, Peter et al., 1967, and Rosner, 1968) or contaminating viral agents (McKercher, 1968, Peacock, 1968, Tamoglia, 1968). Evaluation of BVD vaccines presented some difficulties because experimentally produced disease is mild whether challenged via the respiratory route (Mills et al.,

1968, Tyler et al., 1968) or through the intravenous route (Malquist, 1968, Tyler et al., 1968). A soluble antigen vaccine was investigated by Fernelius et al., 1971b, and vaccinated calves developed high titer of neutralizing antibodies against BVD virus and compared favorably with that produced by an attenuated live-virus vaccine and they recommended the use of these inactivated vaccines to reduce the number of BVD virus carriers. Complications after vaccination with BVD were attributed to either a deficiency of the immune system or the cattle were in the incubative stage at the time of vaccination, (McKercher et al., 1968, Peter et al., 1967). Phillips et al., 1975, have attenuated BVD in continuous porcine cell line with good immunization results and without post-vaccinal reactions.

From the literature review it is evident that there exist many strains of BVD virus, and investigative work on the antigenic relationship among these strains is lacking. Antigenic differences of significant nature may possibly change the immunological properties which may require serious considerations for the production of a vaccine using a particular strain or a combination of viruses.

Therefore, an attempt was made to study the antigenic similarities and differences among three prototype viruses and four field isolates (Minnesota) in this report.

SECTION III

MATERIALS AND METHODS

MATERIALS AND METHODS

Viruses Used

NADL (ATCC): The cytopathogenic strain was isolated in January, 1962 by Gutekunst and Malquist from a clinical case of BVD virus occurring in the National Animal Disease Laboratory (NADL) Holstein-Friesian herd. This strain was characterized by Gutekunst and has been maintained at -75° C since its isolation as original infected tissues and as various passages in embryonic bovine kidney (EBK) cells. In this laboratory it has been passaged into Madin-Darby bovine kidney (MDBK) cell cultures and kept at -70° C.

Oregon (C24V) Strain: The cytopathogenic strain, Oregon (C24V) produces a distinctive type of vacuolization in susceptible EBK cells, and which was isolated by Gillespie et al., 1960 from Oregon cattle. It is propagated in MDBK cell cultures and kept at -70° C.

Singer (Maryland): The strain was isolated in Maryland and propagated in MDBK cell cultures and kept at -70° C.

LlP 10 x 175 (M_1)*: Isolated from a hereford calf in 1962, one of seven which died out of a herd of 13 calves. It was passed 10 times on bovine fetal endocardial cells and once on bovine turbinate cells. It is propagated three times on MDBK cells and kept at -70° C.

---

*Minnesota virus isolates kindly provided by Dr. D. W. Johnson.

MJ P725 IV 75 (M_2)*: Isolated from one Holstein in June, 1961, which was ill with BVD, from a herd of 66. This virus has been passed seven times on bovine fetal endocardial cells and one passage on turbinate cells. It has been passed three times on MDBK cell cultures and kept at -70° C.

74-1865 (M_3)*: This virus was isolated in December, 1974 from the spleen and lung from a feeder calf that died with clinical signs of diarrhea and respiratory disease. The calves had been vaccinated with tri-valent (PI_3, IBR, BVD) 1-1/2 months prior to becoming ill. The isolation was made on bovine turbinate cells. It was propagated three times on MDBK cell cultures and kept at -70° C.

75-1842 (M_4)*: This virus was isolated in December, 1975 from the nasal swab taken from a calf ill with a disease clinically diagnosed as BVD. It was isolated after four blind passages on bovine turbinate cells and three times on MDBK cell cultures and kept at -70° C.

The virus isolates were passaged 3-4 times in MDBK monolayer cell cultures grown either in half-gallon roller bottles (Bellco Glass, Inc., Vineland NJ 08360) or in 32-oz. glass prescription bottles. The cultures were inoculated at a multiplicity of approximately 1 PFU/cell and incubated at 36° C. Cells were harvested after 48-96 hours post virus inoculation when 90% or more of cells showed cytopathic effect (CPE) with the exception of M_2 which was harvested after 120-192 hours.

Infectious tissue culture fluids were subjected to three cycles of freezing at -70° C in an ultra-freezer (Revco, Inc., Model ULT 1285-B-5) and clarified by low speed centrifugation in an International refrigerator centrifuge model PK-2, 20 minutes at 2000 g at 5° C. Cell free infectious fluids were dispensed in 100 ml. plastic bottles and kept frozen at -70° C as stock viruses.

Cell Cultures and Media: The Madin-Darby bovine kidney (MDBK) cell line, originally established by Madin and Darby (1958) was used in the present studies. Cells were produced in roller or prescription bottles seeded with cell suspension at the rate of 6×10^4-2×10^5 cells/ml. Roller bottles were incubated in a walk-in incubator (Forma Scientific) on a roller apparatus (Bellco Glass, Inc., Vineland, NJ 08360) at a speed of 1/2-1.0 rpm. Complete monolayers were formed two to three days post-seeding. Cells were washed twice with phosphate buffered saline (PBS), and dispersed with Trypsin-EDTA mixture 0.05% (w/v) trypsin (1:250, Difco) and 0.02% w/v) EDTA, pH 7.2 . The trypsin mixture was spread over the monolayer, drained and minimal essential medium Eagle's (Grand Island Biological Co., Grand Island, NY) containing 10% inactivated horse serum was added. Cells were counted in a hemocytometer and then seeded in bottles. Cells were frozen in dimethyl-sufoxide $(CH_3)_2 SO_4$ (Mallinckrodt Chemical Works, St. Louis, Mo. 63160) (10%) for storage.

Petri dishes (60 x 15 mm., Falcon, 1950 William Drive, Oxford, CA 93030) were seeded with 1.1-1.2 million cells each to obtain monolayer cultures in 18-24 hours, post-seeding.

BVD Virus Assay: Virus assay procedure was essentially similar as described for animal viruses by Dulbecco (1952) with some modifications. The procedure was later described for BVD virus by Kniazeff et al., (1963) and Singh (1969), Fernelius (1967), Gratzet et al., (1967), Hafez et al., (1972a).

MDBK cell cultures were propagated in 60 x 15 mm. petri dishes. Serial 10-fold virus dilutions were made in MEM containing 5% inactivated horse serum. Growth medium was removed from the petri dishes containing MDBK cell monolayers and 0.2 ml. each of the serial virus dilution was inoculated into duplicate cultures. Two uninoculated petri dishes were used as control. Virus adsorption was allowed to take place for 1-1/2-2 hours at 37° C in 5% CO_2 in a humidified incubator (Hotpack, Philadelphia, PA). An overlay containing a mixture of 2% purified agar and 2X MEM with 5% horse serum was added to each dish (8 ml.). After the agar overlays solidified, dishes were reincubated as before.

Five to six days later 3 ml. of media agar mixture containing 0.015% neutral red was added to each dish to stain the monolayers. Plaques were observed the following day and virus titers were determined as plaque-forming units per ml. (PFU/ml).

BVD Virus Concentration and Purification: Monolayers of MDBK cell cultures were inoculated with virus and harvested when maximum cytopathic effect was observed. After three cycles of freezing and thawing, the released virus present in the supernatant culture fluids (stock virus) was concentrated by centrifuging at 80,000 g for five hours in SW 27 swinging-bucket rotor, Beckman L2-65B. The resulting pellet was suspended in tris-saline (0.01 M tris, 0.1 M NaCl and pH 7.2). The suspended viruses were sonicated three times for 15 seconds at 0^{o} C in an ultrasonic disintegrator (Model W185, Heat System-Ultrasonics, Inc., Plainview, L.I., NY). The sonicated viruses were treated with trypsin (10-20 ug/ml.) for 30 minutes, trypsin inhibitor for 20 minutes and then RNase and DNase (20 ug/ml.) for 30 minutes at 37^{o} C, according to the methods of Burki, 1965; Gutekunst and Malquist, 1965; Fernelius, 1968; and Minocha and Consigli, 1967, 1968, with modifications. The enzymes were removed by ultracentrifugation at 100,000 g for 1/2 hour in 50.1 rotor (Beckman) and the virus pellet was washed in tris-saline and briefly sonicated as mentioned above. The sonicated viruses were layered on top of 10% sucrose (w/v) in TEN buffer: (0.01 M tris, 0.001 M EDTA 0.1 M NaCl, adjusted to pH 7.4 with HCl) in 5 ml. cellulose nitrate tubes and according to the procedures used by Fernelius, 1968; Maess et al., 1970; Horzinek et al., 1971; Parks et al., 1972 and Scott et al., 1974, with some modifications. The tubes were

spun at 100,000 g for 2 hours in SW 50.1 rotor in Beckman L2-65B ultracentrifuge. The sedimented viruses were resuspended in tris-saline or TEN buffer.

The resuspended viruses were briefly sonicated as described previously and carefully layered on top of a preformed potassium tartrate (Fisher Scientific) gradient. The gradients 10-30% (w/v) had been formed in TEN buffer using a gradient maker (Buchler Instruments, Fort Lee, NY); a 1.0 ml. cushion of 38% (w/v) potassium tartrate solution was used at the bottom of the tube (17 ml. cellulose nitrate tubes, Beckman centrifuge tubes) according to Maess et al., 1970. The tubes were spun at 80,000 g for three hours in Beckman ultracentrifuge in SW27 rotor. Likewise, 50% potassium tartrate (w/v) and 30% glycerol (v/v) were used except that the centrifugation time was 20 hours. Similarly, $CsCl_2$ was used according to the method described by Fernelius (1968) and Ritchie et al. (1969). Discontinuous $CsCl_2$ density gradients were prepared by the addition of 0.5 ml. of 1.4, 1.35, 1.30, 1.25, 1.20 and 1.15 solutions prepared from a stock solution of 1.6 density. Virus preparation (0.5 ml.) was layered on top of the gradient and centrifuged at 80,000 g for 12-24 hours using SW27 rotor.

Fractions were collected through a hole in the bottom of the tube using a fraction collector (Model 327, Instrumentation Specialties Co.), and Buchler piercing units. Each

fraction was transferred to number 1/4 inch diameter dialyzer tubing (Fisher Scientific Co.) and dialyzed in tris-saline for 24 hours using a multiple dialyzer (Pope Scientific, Inc.). The dialyzing fluid was changed 3-4 times at 4^{o} C.

Fractions were plaque assayed and those showing the highest infectivity titers were pooled and used for rabbit inoculation.

BVD Virus Density and Infectivity: The refractive indices of fractions from $CsCl_2$ were read in a Bausch and Lomb Abbe' 3L refractometer and buoyant densities were determined by the method of Ifft et al., 1961. Fractions were plaque assayed for infectivity determination.

Protein Determination: Protein was measured by the method of Lowry et al. (1951) using crystalline bovine serum albumin as a standard.

Electron Microscopy: Samples of virus-containing fractions from potassium tartrate gradients were negatively stained with 1% (w/v) sodium phosphotungstate, pH 7.0 (according to Miller, 1974 and Watson et al., 1963). Examination was carried out using Phillips EM 102 electron microscope at magnifications of 13,800 to 70,000 in the Division of Biology at Kansas State University. One drop of virus preparation was layered onto carboned formvar grids and the excess virus was removed with filter paper by touching the side of the grid, and one drop of 1% Na-phosphotungstate was added to the grid and after a few

seconds air dried and studied in the electron microscope.

Neutralization Tests

Production of Rabbit Hyperimmune Sera: Two albino rabbits (6-8 weeks) were used for each BVD virus isolate for the production of hyperimmune serum. Rabbits were inoculated intravenously with purified virus preparations containing 10^5-10^7 PFU/ml. Animals were given 0.2, 0.3, 0.5, 0.75 and 1.0 ml. of virus over a month period at weekly intervals. Preimmunization serum was collected to be used as control serum for neutralization tests.

Rabbits were bled by ear vein under negative pressure (about 1 lb.) and blood was kept for one hour at room temperature. After the clot had formed, the blood was transferred to a refrigerator overnight. Serum was collected and centrifuged at 1000 g for 10 minutes. The sera of each of the two rabbits inoculated with the virus were pooled (inactivated at 56° C for 30 minutes) and the antibody titer was determined by the plaque reduction procedure.

Titration of Antisera with Homologous Virus: All BVD virus antisera and normal rabbit sera (pre-inoculation) were diluted in MEM plus 5% horse serum. Dilutions were 1/50-1/400. Each virus was diluted to contain 10^5 PFU/ml. Dilutions of antiserum (0.4 ml.) were mixed with equal volume of the homologous virus, well mixed and incubated overnight at 4° C. Each serum-virus mixture was diluted from

10^{-1} to 10^{-3} whereas virus alone and virus plus normal rabbit sera (control) were diluted from 10^{-1} to 10^{-4} and plaque titered.

Cross-Neutralization Tests: The antigenic relationship of BVD virus strains was investigated by the serological cross-neutralization reactions. Each BVD virus antiserum was cross-reacted with the seven BVD virus isolates. The corresponding normal rabbit serum was used as control.

Each BVD virus antiserum was diluted either from 1/50 to 1/400 or 1/200 to 1/1600 depending upon the serum neutralization titers obtained in homologous serological reaction. The highest dilution of the antiserum which completely neutralized the homologous virus was taken as the lowest dilution in the cross neutralization tests. Each antiserum dilution (0.4 ml.) was mixed with equal volume of each of BVD virus containing 4×10^4 PFU's. In each test the BVD viruses with normal rabbit sera were used to serve as controls. Serum-virus mixture and viruses with media were incubated at 4° C overnight. Serum-virus mixtures were diluted from 10^{-1} to 10^{-3}. BVD viruses alone or BVD viruses plus normal rabbit sera were diluted from 10^{-1} to 10^{-4}. All samples were plaque assayed as described.

The data on cross neutralization reactions was studied by Archetti-Horsfall formula and subjected to computer analysis for antigenic relationship among the virus isolates tested.

SECTION IV

RESULTS

RESULTS

BVD Virus Plaque Characteristics: The plaque morphologies of NADL, Singer, M_3 and M_4 appeared essentially indistinguishable. NADL, M_4 and Singer had the largest plaques while M_1 and M_2 had relatively smaller plaques. C24V plaques showed characteristic sharp, circular clear-cut margin, while the other isolates had plaques with diffusable boundaries (Figure 1).

BVD viruses plaqued on MDBK cell monolayers and stained with neutral red six days after inoculation produced different plaque size (Figure 1 and Table 1). Statistical analysis of the plaque size data at 5% level of significance using t-test (Table 1) demonstrated that there was no significant difference in plaque size between M_1 and M_2 (1.8-2.2 mm.); C24V and M_3 (2.9-3.3 mm.); M_3, Singer and NADL (3.7-4.1 mm.); and NADL and M_4 (4.1-4.3 mm.). A significant difference in plaque size was observed between M_1 and C24V; C24V and Singer and Singer and M_4.

BVD Virus Concentration and Purification: Table 2 presents the results of BVD virus concentration and purification. Ultracentrifugation of the crude virus showed 92% recovery of the virus with 11 fold purification. Treatment of sedimented virus with enzymes (DNase, RNase and Trypsin) demonstrated 76.9% recovery of viral infectivity with 108 fold purification. The final purification steps of passing the virus through 10% sucrose followed by K-tartrate gradient centrifugation resulted

in 53.8 and 42.3% recovery of virus, respectively. The fold purification in these two steps was 606 and 1,000 respectively. More than 99% of the original protein was removed after potassium tartrate gradient centrifugation. The specific activity (total PFU/total protein) had increased from 1.1×10^3 in the crude virus to 1.1×10^6 in the last step of purification.

Buoyant Density of BVD Virus: A typical density and infectivity distribution of BVD in $CsCl_2$ gradient is represented in Figure 2. From the refractive indices measurements of $CsCl_2$ fractions the buoyant density of BVD virus was determined using the procedure of Ifft et al., 1961 at room temperature. BVD virus (NADL) density ranged from 1.13 to 1.17 g/cm^3 with the highest peak of infectivity at a density of 1.15 g/cm^3.

Electron Microscopy: Purified preparations of BVD virus showed different forms of viral particles by electron microscopy. This variation may be due to the properties of BVD virus or the result of different treatments during manipulations of virus preparations. At least four virus populations were observed: enveloped full, enveloped empty, naked full and naked empty particles (Figure 3).

Full viral particles displayed three parts: (1) an outer structure, the envelope (2) the capsid and (3) the core, occupying a central position which seems to be penetrated by the stain in most of the preparations. Cores appeared to be hexagonal. The capsids displayed a similar shape as the core

and appeared to be hexagonal. They seem to be composed of a number of elongated capsomers which are regularly arrayed and are not closely packed (Figure 3B). Capsid diameter of viruses (Figure 3 and Table 3) was: NADL 76-163 nm., with mean 120.1 nm; C24V 82-181 nm. with a mean of 105.8 nm; and Singer 66-152 nm. with a mean 107.8 nm.

Viral envelopes with varying size and shape in most of BVD virus preparations were observed to enclose the capsid (Figure 3). BVD NADL had an envelope size of 27-58 nm. across with a mean of 38.5 nm. C24V had a size of 12-35 nm. across with a mean 25.2 nm., and Singer had an envelope size of 26-58 nm. across with a mean of 36.8 nm. NADL envelope seemed more stable than that of C24V and Singer as very few naked viral particles were observed. Viral envelopes showed differences in sizes, but no significant difference between NADL and Singer envelopes was observed (Table 3). However, envelopes of NADL and Singer isolates when compared with that of C24V which is smaller and differed significantly in size (Table 3). This may have relationship with the differences observed in serological cross reactions among these viruses.

Serum Neutralization (SN) Tests:

Titration of Homologous Antisera: Antisera prepared against seven isolates of BVD virus demonstrated different titers when reacted with homologous virus containing approximately 4×10^4 PFU (Table 4). NADL, Singer and M_3 showed

complete neutralization of homologous viruses at 1/200 dilution of antiserum. Antisera against $C_{24}V$, M_1 and M_4 maximally neutralized the corresponding viruses at 1/50 dilution and M_2 at 1/100 dilution. Normal rabbit sera did not neutralize BVD virus isolates.

Cross Neutralization Tests: Table 4 also presents the serological cross reactions between BVD antisera and the virus isolates. Singer, M_1 and M_4 are strongly related to NADL as indicated by <8% survival of the viruses tested against NADL antiserum (1/1600 dil.). The percentage survival of $C_{24}V$, M_2 and M_3 virus isolates was 62, 100 and 34%, respectively, with NADL antiserum. The degree of relationship of $C_{24}V$ with the other virus isolates was variable as shown by survival rates of 4 to 46% with $C_{24}V$ antiserum (1/400 dil.). NADL and M_4 showed much greater degree of relationship (1% survival) with anti-Singer (1/1600 dil.) than did M_1 and M_2 (10% survival) on the basis of their reaction with anti-Singer. However, 100% survival of $C_{24}V$ and M_3 viruses with anti-Singer indicated lack of antigenic relationship between these viruses. The cross reaction using M_1 or M_2 antisera and all other BVD viruses showed great heterogeneity in antigenic relationship as indicated by survival of viruses ranging from 11-100%. M_3 showed the least relationship, if any, with BVD viruses as shown by virus survival rates of more than 52% when cross reacted with anti-M_3 (1/1600 dilution). NADL, Singer, M_1 and M_2 viruses

strongly cross reacted with M_4 antiserum (survival <8%). A weak cross reaction of M_3 and C24V with M_4 antiserum (16-49% survival) demonstrated a low degree of relationship between these viruses. These data of SN studies were also graphed and presented as Figure 4.

Antigenic Relationships Among Virus Isolates on the Basis of Archetti-Horsfall Formula: This method had been used for the antigenic analysis of influenza viruses by Archetti and Horsfall in 1950; for classification of bovine enteroviruses in 1965 by LaPlaca et al., and by Castrucci et al., 1968a, b for grouping strains of BVD virus. The geometric mean of the ratio R_1 found on dividing the heterologous titer obtained with virus 2 by the homologous titer obtained with virus 1; and the ratio R_2 found on dividing the heterologous titer obtained with virus 1 by the homologous titer obtained with virus 2 is given by the equation: $R = \sqrt{R_1 + R_2}$. (R) is the extent of the antigenic difference between two viruses when both agents and both antisera are used in a cross-serological reaction. Ratio R is 1 for the homologous antigen-antiserum reaction. Ratio of ≤2 when two virus isolates are cross reacted signifies high degree of antigenic relationship. The data on the basis of R values of BVD virus isolates is presented in Table 5. NADL, Singer, M_1 and M_4 fall into one antigenically related group, since all of them have R values of <2 in cross-neutralization tests, while C24V, M_2 and M_3

each form a group by itself.

Figure 5 summarizes the antigenic relationship between BVD virus isolates. In general, a two-way relationship exists between NADL, Singer and M_4; and between NADL, M_1 and M_4. C24V is related to M_1 and likewise M_2 is to M_4. M_3 had no demonstrable serological relationship with any of the other six BVD viruses.

Statistical Analysis: The data obtained in serum neutralization tests was also subjected to computer analysis* (Tables 6-8) for determination of antigenic relationships among virus isolates. The data has been subjected to computer analysis of variance using the model:

$y = \mu + si + vj + dk + svij + sdik + ijk$; where Y = percentage survival, μ = over-all mean, si = antiserum i main effect, vj = virus j main effect, dk = dilution k main effect, svij = effect of antiserum i as applied to virus j, sdik = effect of antiserum i as applied at a dilution k. Since the antiserum dilution interaction (sd) turned out not to be significant, the results presented here are based on the model:

$y = \mu + si + vj + dk + svij + ijk$; where ijk is an error term. The results of this analysis of variance are summarized in Tables 6-8.

---

*Computer analysis was done by Dr. Loschcke, Christian in the Department of Statistics, KSU.

F-values obtained in Tables 5-6 were analyzed as follows: (1) the F-value of 45.70 shows that the antisera differs significantly from each other at the 0.0001 level of significance. The comparison of the average percentage survival of all BVD viruses per antiserum is shown in column (1) (Table 7). M_1 antiserum is the least effective antiserum followed by M_3 antiserum. NADL and Singer antisera, on the average, are the most effective in their neutralizing ability. (2) the F-value of 17.11 means that BVD viruses are differently neutralized by all antigens, e.g., C24V virus has the highest survival rate (with 44.84% survival) followed next by M_2 virus (30-90.5% survival (Table 7). NADL and M_4 viruses are very easily neutralized leaving 15.953% and 11.479% survival, respectively. (3) the F-value of 16.88% in the analysis of variance table means that different dilutions have different effects on BVD virus isolates (Table 7). The fact that 1/50 and 1/100 and also 1/100 and 1/200 dilutions are equally effective is probably due to the fact that some antisera completely neutralized a given BVD virus at both dilutions. (4) The F-value of 7.28 in the analysis of variance table means that BVD antisera differ in their neutralizing activity towards the different viruses. The average percentage survival of each BVD virus and antiserum are given in Table 8.

SECTION V

TABLES AND FIGURES

TABLE 1. RELATIVE PLAQUE SIZE (DIAMETER IN MM) OF BVD VIRUS ISOLATES GROWN ON MDBK CELL CULTURES AND STAINED WITH NEUTRAL RED 6 DAYS POST INFECTION

VIRUS	NO. OF PLAQUE MEASURED	PLAQUE SIZE DIAMETER MEAN (MM)	DIAMETER RANGE (MM)	STANDARD DEVIATION (MM)	COEFFICIENT OF VARIATION (%)
M_2	5	1.8 *	1.3 - 2.6	0.5	27.8
M_1	10	2.2 *	1.3 - 3.0	0.5	22.7
$C_{24}V$	19	2.9 *	2.0 - 3.9	0.6	20.7
M_3	20	3.3 * *	2.0 - 5.3	0.8	24.2
SINGER	13	3.7 *	2.0 - 5.3	1.3	35.1
NADL	19	4.1 * *	3.0 - 5.9	0.9	21.9
M_4	24	4.3 *	2.6 - 6.6	1.3	30.2

TABLE 2. CONCENTRATION AND PURIFICATION OF BOVINE VIRAL DIARRHEA VIRUS (SINGER-MARYLAND STRAIN)

SAMPLE	TOTAL VIRUS (PFU)	% VIRUS RECOVERY	TOTAL PROTEIN UG	% PROTEIN RECOVERY	SPECIFIC ACTIVITY TOTAL PFU/ TOTAL PROTEIN	FOLD PURIFI-CATION
CRUDE VIRUS	2.6×10^9	100	2.34×10^6	100	1.1×10^3	1
SUPERNATANT	1.2×10^6	0.05	2.16×10^6	92.0	0.5	-
ULTRACENTRIFUGE SEDIMENT	2.4×10^9	92.3	1.82×10^5	7.8	1.3×10^4	11
ENZYME TREATED (DNASE, RNASE AND TRYPSIN)	2.0×10^9	76.9	1.67×10^4	0.7	1.19×10^5	108.8
10% SUCROSE	1.4×10^9	53.8	2.1×10^3	0.09	6.6×10^5	606.0
K-TARTRATE GRADIENT (10-30%)	1.1×10^9	42.3	1.0×10^3	0.04	1.1×10^6	1000

TABLE 3. MEASUREMENTS FROM ELECTRON MICROSCOPY OF BVD VIRUS, ITS CAPSID AND ENVELOPE UNDER PTA

OBJECT	NO. OF PARTICLES MEASURED	MEAN VALUE (NM)	STANDARD DEVIATION (NM)	COEFFICIENT OF VARIATION (%)
NADL				
CAPSID	12	120.2	7.7	6.4
ENVELOPE	10	38.6	3.0	7.7
$C_{24}V$				
CAPSID	8	105.9	9.5	8.9
ENVELOPE	8	25.2	3.7	13.7
SINGER				
CAPSID	18	107.9	6.3	5.8
ENVELOPE	8	36.9	3.5	9.4

TABLE 4. CROSS NEUTRALIZATION OF BVD VIRUS ISOLATES IN MDBK CELL CULTURES

VIRUS	% SURVIVAL (PFU)*							
	ANTI-NADL				ANTI-$C_{24}V$			
	1/200	1/400	1/800	1/1600	1/50	1/100	1/200	1/400
NADL	0	0.074	0.147	1.22*	0.8	1.0	2.5	6.5
$C_{24}V$	20	62	33	62	0.8	7.5	20	23
SINGER	0	0.06	0.3	0.8	0.7	1.8	3.1	10.3
M_1	0	0	0.6	7.7	1.8	2.1	4.7	20
M_2	0.6	3.5	68.8	100	0.5	1.2	18.2	30
M_3	1.8	8.6	9.1	34.1	18.8	27.5	36.3	46.9
M_4	0	0.2	0.7	4.3	1.1	3.9	4.7	10

$$* \% \text{ SURVIVAL} = \frac{\text{TITER OF VIRUS WITH ANTISERUM}}{\text{TITER OF VIRUS WITH NORMAL RABBIT SERUM}} \times 100$$

TABLE 4. (CONTINUED)

VIRUS	% SURVIVAL (PFU)							
	ANTI-SINGER				ANTI-M_1			
	1/200	1/400	1/800	1/1600	1/50	1/100	1/200	1/400
NADL	0	0	0.07	0.2	29	56	50	25
$C_{24}V$	30.7	71.4	50	100	39.4	63.6	39.4	54.5
SINGER	0	0	0.1	0.6	100	93	100	86.7
M_1	0	0	6	10	12	37	70	100
M_2	0.05	8.3	10	30	43.6	49.1	91	50
M_3	5.8	35.8	100	100	24.2	38.4	26.3	42.1
M_4	0	0.03	0.3	0.63	11.2	14.7	13.5	22.4

TABLE 4. (CONTINUED)

	% SURVIVAL (PFU)											
	ANTI-M_2				ANTI-M_3				ANTI-M_4			
	1/100	1/200	1/400	1/800	1/200	1/400	1/800	1/1600	1/50	1/100	1/200	1/400
NADL	0.7	2.3	7.5	11.6	30	39	59	90	0	0	0	0.08
$C_{24}V$	17.8	19.2	31.5	47.9	41.6	41.6	58.3	100	45.9	39.8	44.9	49.0
SINGER	0.9	5	10	25	36	50	44	52	0	0.05	0.1	0.6
M_1	1.7	12.1	24.3	45.7	23.6	33.6	100	80	0	0	0.4	1.4
M_2	0.3	0.4	1.0	8.1	66.7	73.3	75	100	0	5.9	6.5	8.2
M_3	16.5	15.6	21.3	73.9	0.1	2.6	2.7	16.3	5	9.4	6.3	16.3
M_4	1.8	3	12.7	12.7	20	30.8	50	65.4	0.1	0.3	1.0	2.7

TABLE 5. SEROLOGIC CORRELATIONSHIPS AMONG BVD VIRUS ISOLATES

ANTISERUM	VIRUS ISOLATE AND R VALUES*						
	NADL	$C_{24}V$	SINGER	M_1	M_2	M_3	M_4
NADL	1	2.9	0.5	0.95	12.9	14.4	0.4
$C_{24}V$	2.9	1	6.4	0.8	3.9	3.3	6.8
SINGER	0.5	6.4	1	2.5	11.5	11.2	0.9
M_1	0.95	0.8	2.5	1	2.3	5.3	0.63
M_2	12.9	3.9	11.5	2.3	1	10.5	1.3
M_3	14.4	3.3	11.2	5.2	10.5	1	4.5
M_4	0.4	6.8	0.9	0.63	1.3	4.5	1

* R (EXTENT OF THE ANTIGENIC RELATIONSHIP AMONG TWO STRAINS) = $\sqrt{R_1 \times R_2}$

$R_1 = \frac{\text{HETEROLOGOUS ANTIBODY TITER (VIRUS 2)}}{\text{HOMOLOGOUS ANTIBODY TITER (VIRUS 1)}}$

$R_2 = \frac{\text{HETEROLOGOUS ANTIBODY TITER (VIRUS 1)}}{\text{HOMOLOGOUS ANTIBODY TITER (VIRUS 2)}}$

TABLE 6. ANALYSIS OF VARIANCE OF THE PERCENTAGE SURVIVAL OF BVD VIRUS ISOLATES IN RECIPROCAL CROSS SERUM NEUTRALIZATION TESTS

	DEGREE OF FREEDOM	SUM OF SQUARE	MEAN SQUARE	F-VALUE
ANTISERUM(S)	6	52,443	8,740.5	45.70 (1)
VIRUS(V)	6	19,636	3,272.67	17.11 (2)
DILUTION	5	16,137	3,227.4	16.88 (3)
SV	36	50,097	1,391.58	7.28 (4)
ERROR	142	27,158	191.25	
CORRECTED TOTAL	195	165,471		

TABLE 7. ANALYSIS OF F-VALUES AT DIFFERENT SOURCES

F-VALUES AND SOURCES

ANTISERUM (% SURVIVAL ALL VIRUSES)		VIRUSES (% SURVIVAL ALL ANTISERA)		DILUTION (% SURVIVAL ALL VIRUSES)	
M_1	58.168	$C_{24}V$	44.648	1/50	9.427 *
M_3	42.819	M_2	30.905 *	1/100	14.698 * *
$C_{24}V$	19.666 *	M_3	27.676 * *	1/200	17.83 *
M_4	17.439 * *	SINGER	23.451 *	1/400	24.014
M_2	17.273 * *	M_1	22.45 * *	1/800	36.842
SINGER	12.741 * *	NADL	15.953 * *	1/1600	48.527
NADL	8.455	M_4	11.479 *		

TABLE 8. MEAN PERCENT SURVIVAL OF BVD VIRUSES BY CROSS-NEUTRALIZATION TEST*

ANTISERUM	BVD ISOLATES						
	NADL	$C_{24}V$	SINGER	M_1	M_2	M_3	M_4
NADL	0	38.0	0	0	36.7	6.8	0
$C_{24}V$	11.5	21.6	12.7	15.9	21.2	41.1	13.7
SINGER	0	56.5	0	0	0.8	53.9	0
**M_1	48.8	57.9	103.7	63.6	67.4	41.5	24.2
**M_2	7.4	31.0	12.2	22.8	4.3	33.7	9.4
**M_3	47.9	53.8	39.1	52.7	72.2	0	35.0
**M_4	8.8	53.6	8.9	9.2	13.9	17.9	9.8

* THE AVERAGES ARE THE LEAST SQUARES ESTIMATES BASED ON THE LINEAR MODEL:

$$Y = U + S_I + V_J + SV_{IJ} + D_K + E_{IJK}$$

WHERE U = THE OVERALL MEAN
S_I = THE EFFECT OF THE ANTISERUM (I)
V_J = THE EFFECT OF THE VIRUS (J)
SV_{IJ} = THE EFFECT OF ANTISERUM (I) ON THE VIRUS (J)
D_K = THE EFFECT OF THE DILUTION (K); E_{IJK} = ERROR.

**MINNESOTA VIRUS ISOLATE

Figure 1. Plaque Formation by Bovine Viral Diarrhea Viruses on MDBK Cells. Seven BVD virus isolates were plaque assayed on MDBK cell cultures. Virus adsorption was done at 37° C in 5% CO_2 for 1 1/2-2 hours in a humidified incubator. Inoculated monolayers were overlayed with 8 ml. of a mixture containing 2% purified agar and 2x MEM with 5% inactivated horse serum. Dishes were reincubated as before and 5 days later were fed with media and agar mixture containing 0.015% neutral red. Plaques were observed the following day and average plaque size was determined. Original plate diameter (5 cm.) was reduced to 3 cm. in the photograph.

THIS BOOK CONTAINS SEVERAL DOCUMENTS THAT ARE OF POOR QUALITY DUE TO BEING A PHOTOCOPY OF A PHOTO.

THIS IS AS RECEIVED FROM CUSTOMER.

THIS BOOK CONTAINS NUMEROUS PICTURES THAT ARE ATTACHED TO DOCUMENTS CROOKED.

THIS IS AS RECEIVED FROM CUSTOMER.

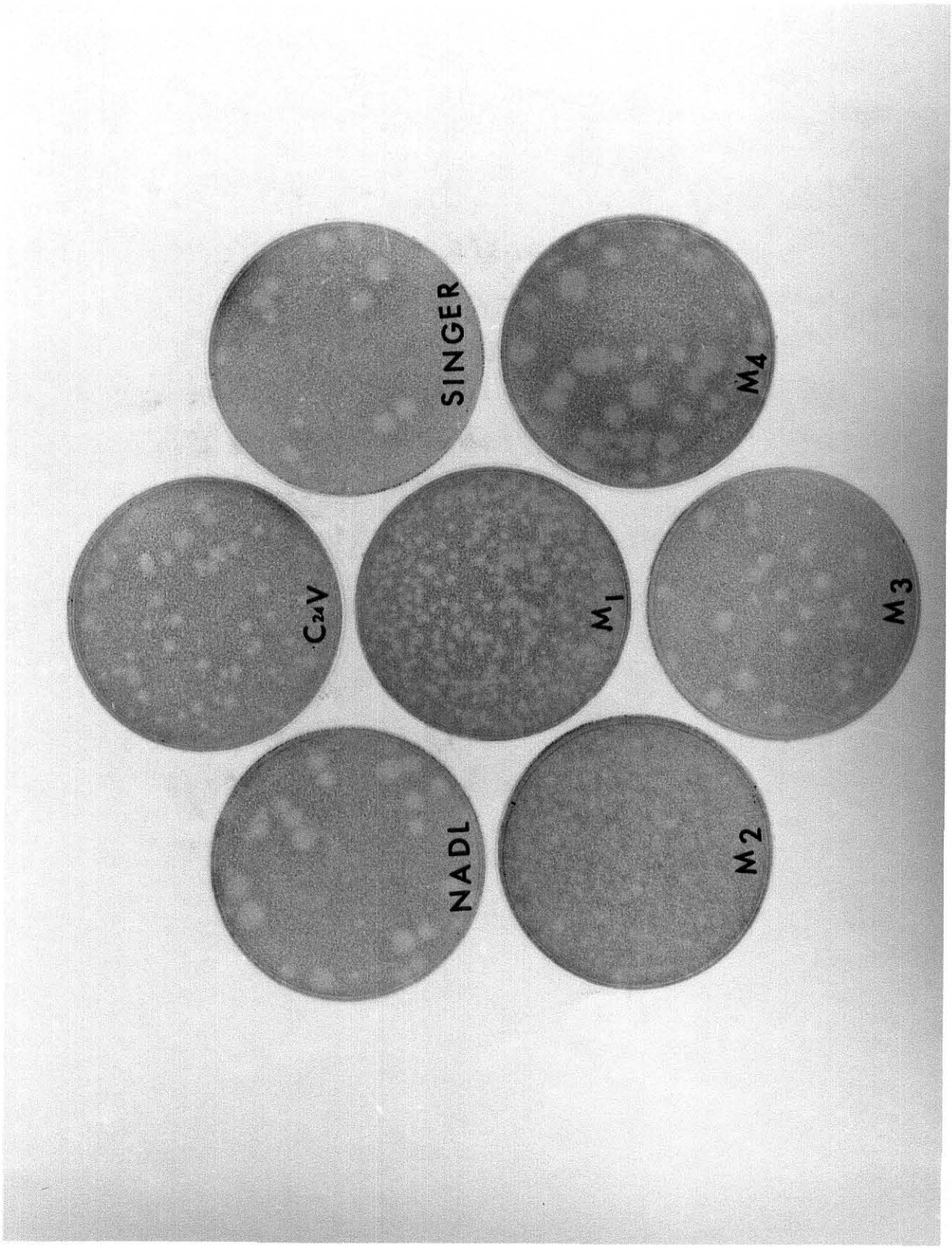

SINGER
M4
C2V
M1
M3
NADL
M2

Figure 2. BVD Virus Density and Infectivity. Density gradient centrifugation of BVD virus (NADL) was done in $CsCl_2$ discontinuous gradient in ten buffer at 80,000 g for 18 hours using SW27 rotor in Beckman L2-65B ultracentrifuge. Fractions were collected, plaqued assayed for infectivity and the density was determined from the refractive indices using Ifft et al., 1961 formula (10.86 x Reading of the refractometer) - 13,4974 . Log virus infectivity (PFU/ml) vs fraction number were plotted. Density (g/ml.) values were plotted for each fraction. Symbols: 0———0, log infectivity (PFU/ml.) △—△, density (g/ml.).

THIS BOOK CONTAINS NUMEROUS PAGES WITH DIAGRAMS THAT ARE CROOKED COMPARED TO THE REST OF THE INFORMATION ON THE PAGE.

THIS IS AS RECEIVED FROM CUSTOMER.

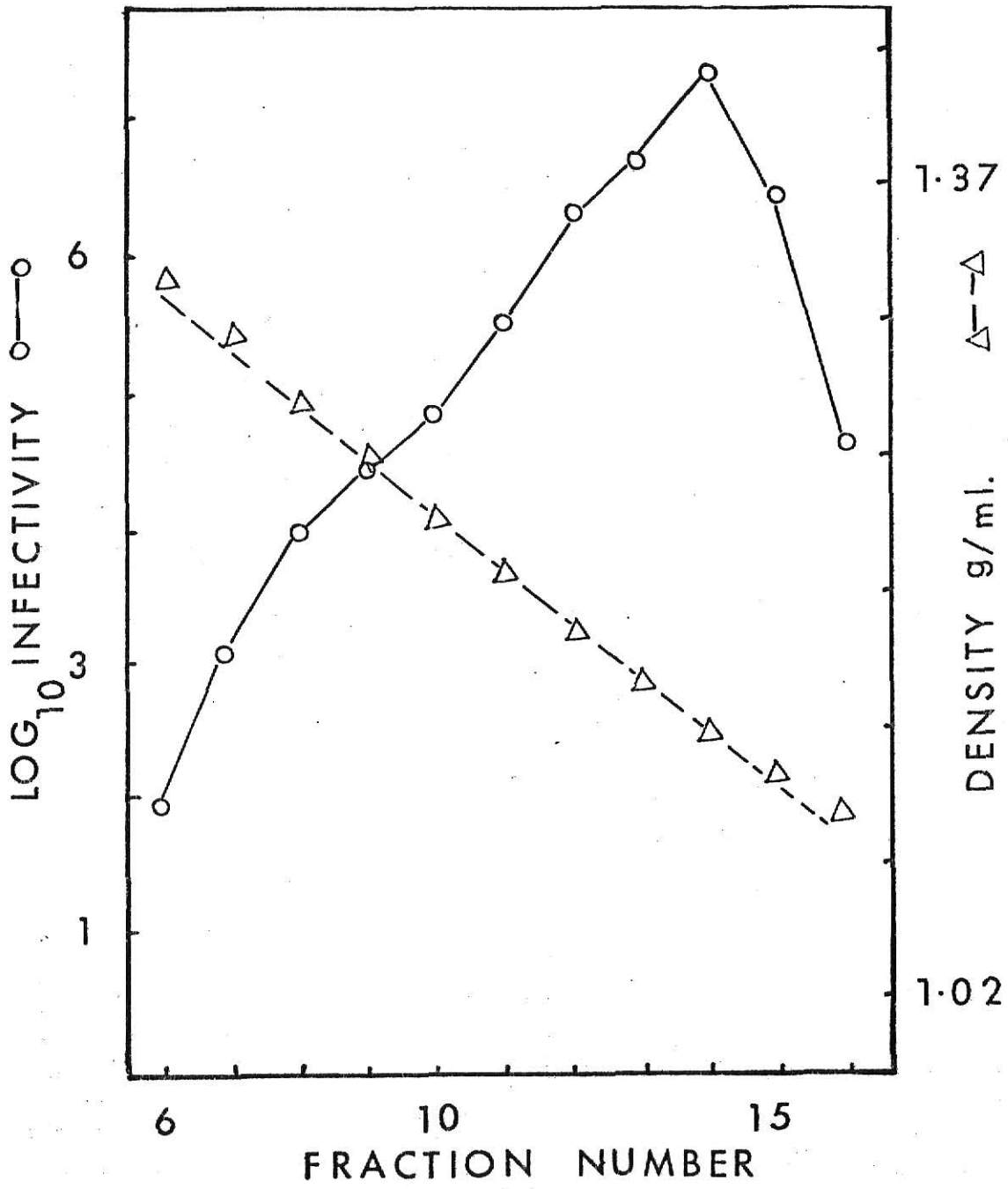

LOG10 INFECTIVITY
6
3
1
1·37
1·02
DENSITY g/ml.
6
10
15
FRACTION NUMBER

Figure 3. Electron Micrographs of Three BVD Virus Isolates. Virus preparations were negatively stained with Na-phosphotungstate.

(i) NADL: Two enveloped, empty virus particles with smooth surface (x 170,800).

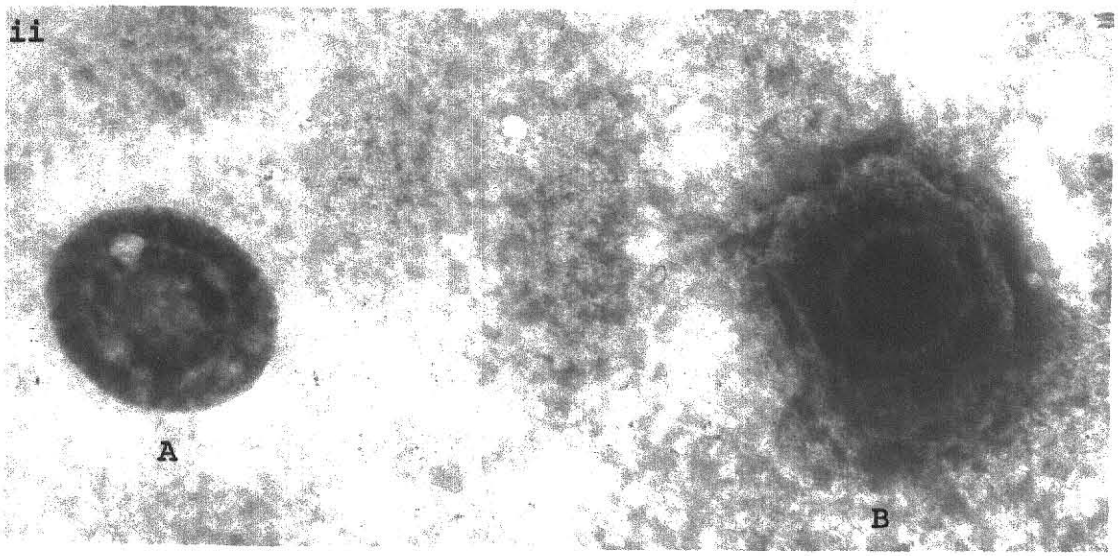
(ii) C24V: A, Partially full, enveloped virus particle. B, Enveloped, empty virus particle (x 184,888).

(iii) Singer: Several naked, empty or partially full virus particles (x 116,666).

i



ii



iii

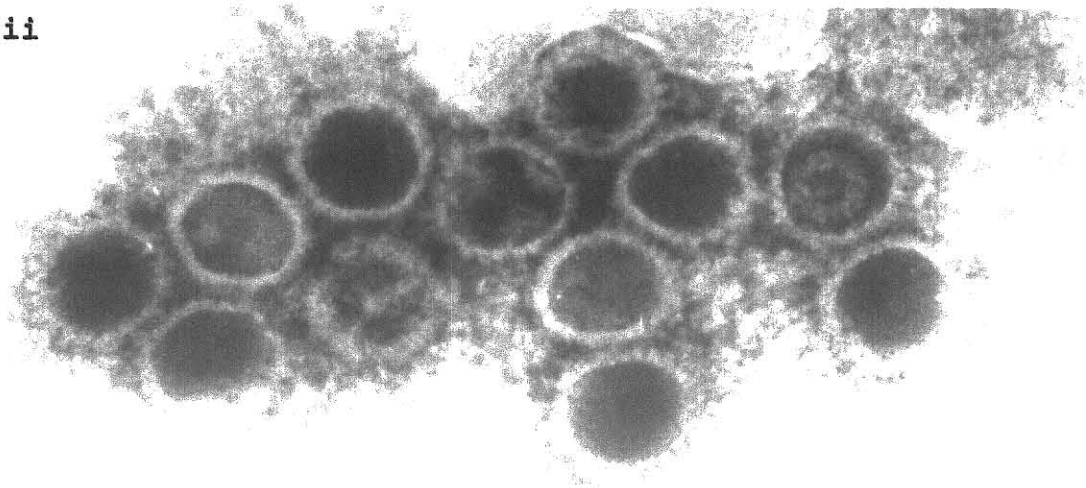


Figure 4. Cross-Neutralization of 7 BVD Virus Isolates. Antisera prepared in rabbits against 7 BVD virus isolates using purified antigens were cross reacted in a constant-virus variable-serum neutralization tests. The dilution of the antibody which completely neutralized the homologous virus (4×10^4 PFU) was taken as the lowest antibody dilution in each cross-neutralization reaction. Equal volume of antibody (0.4 ml.) was thoroughly mixed with equal volume of virus containing 4×10^4 PFU and incubated at 4° C overnight. Virus serum mixtures were plaque assayed on MDBK cell cultures and the percentage survivals of all viruses with each antiserum were plotted against the dilutions. Symbols: > , antiserum, -0-, NADL virus, -△- , C24V virus, X, Singer virus, -□- , M_1 virus, -●- , M_2 virus, -▲- , M_3 virus, -■- , M_4 virus.

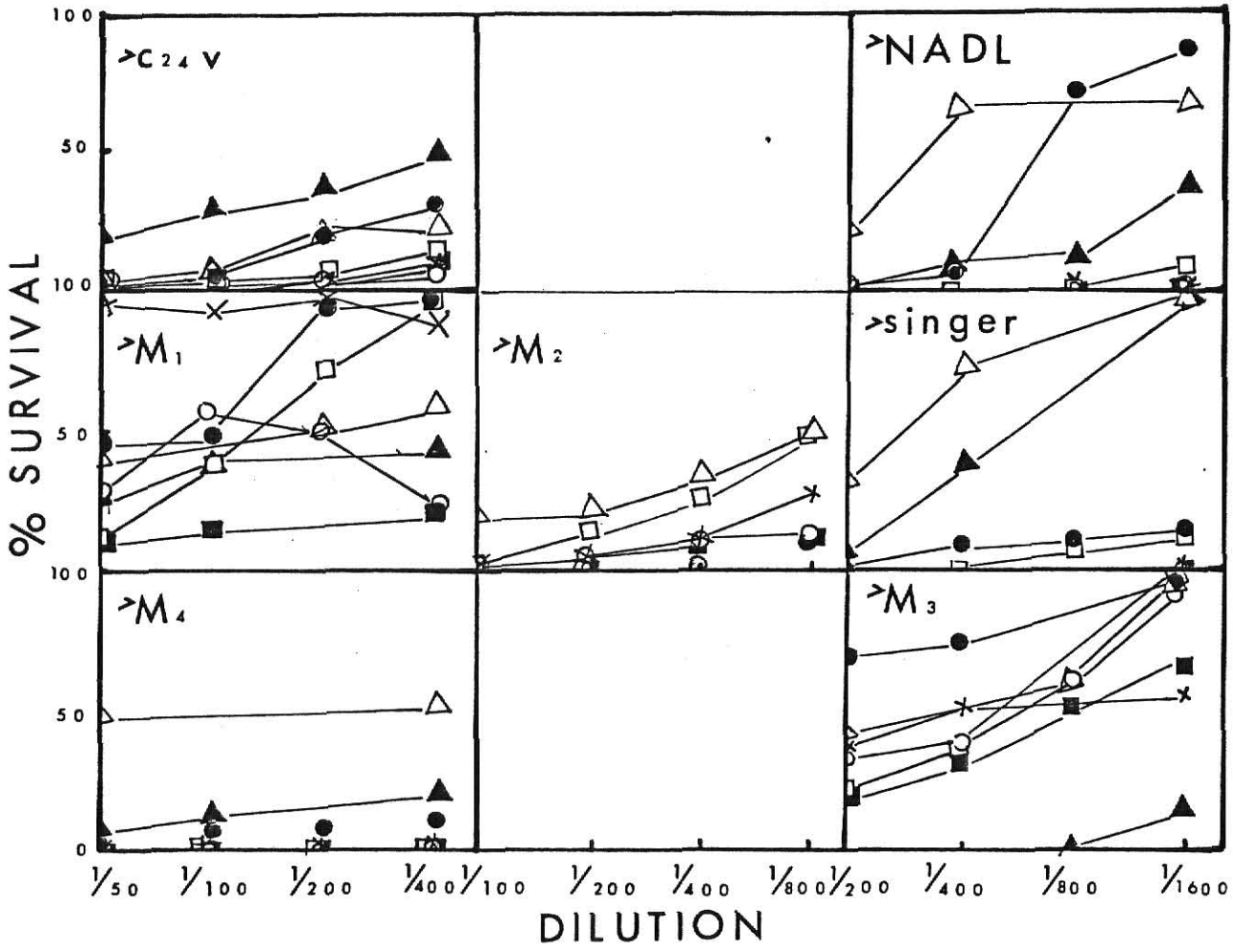

C24V
NADL
M1
M2
singer
M4
M3
% SURVIVAL
100
50
100
50
100
50
0
1/50 1/100 1/200 1/400
1/100 1/200 1/400 1/800
1/200 1/400 1/800 1/1600
DILUTION

Figure 5. Major Antigenic Groups Among BVD Virus Isolates. Cross neutralization tests followed by analyses using Archetti-Horsfall equation ($R = \overline{R_1 \times R_2}$) revealed antigenic relationships between BVD virus isolates tested.

R = Extent of antigenic relationship.

R_1 = Heterologous antibody titer (Virus 2) / Homologous antibody titer (Virus 1)

R_2 = Heterologous antibody titer (Virus 1) / Homologous antibody titer (Virus 2)

For homologous antigenic antibody reactions, R value is 1. R values 2 or 2 represents strong antigenic relationship between the two virus isolates tested and R value 2 may represent a weak antigenic relationship.

Arrows indicate antigenic relationship. NADL, Singer, M_1 and M_4 show a two-way relationship and form a group by themselves. M_2 and C24V each are related to only one of the other isolates while M_3 shows no relationship with any of the isolates tested.

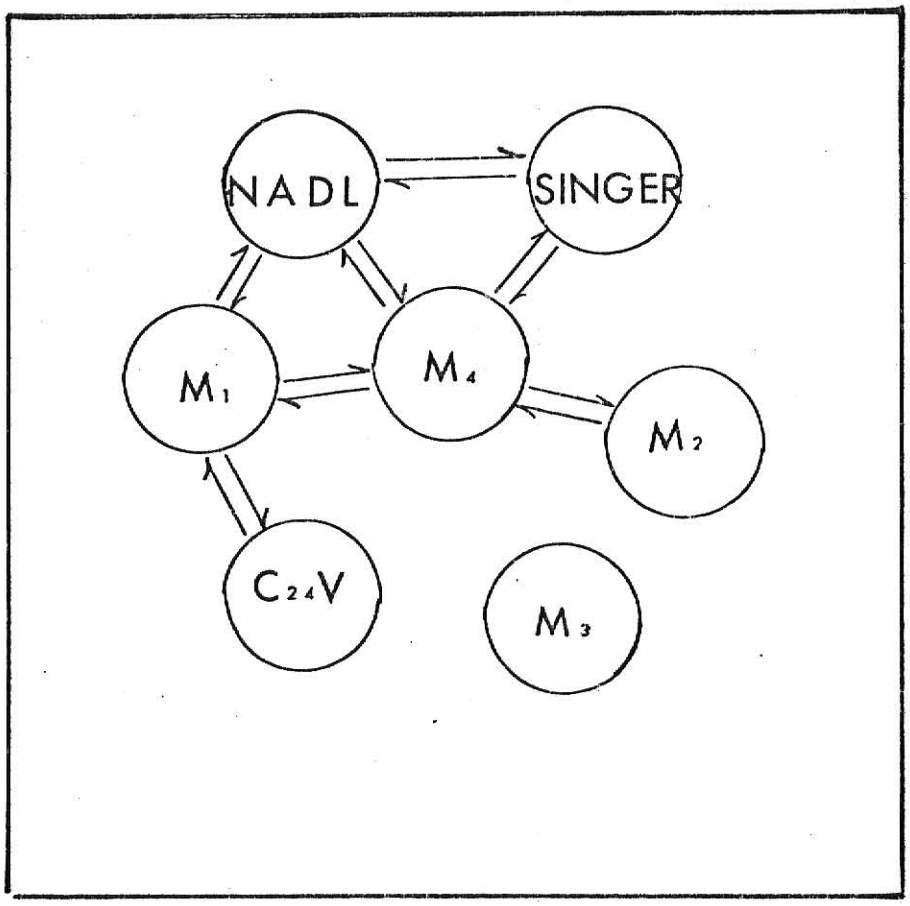
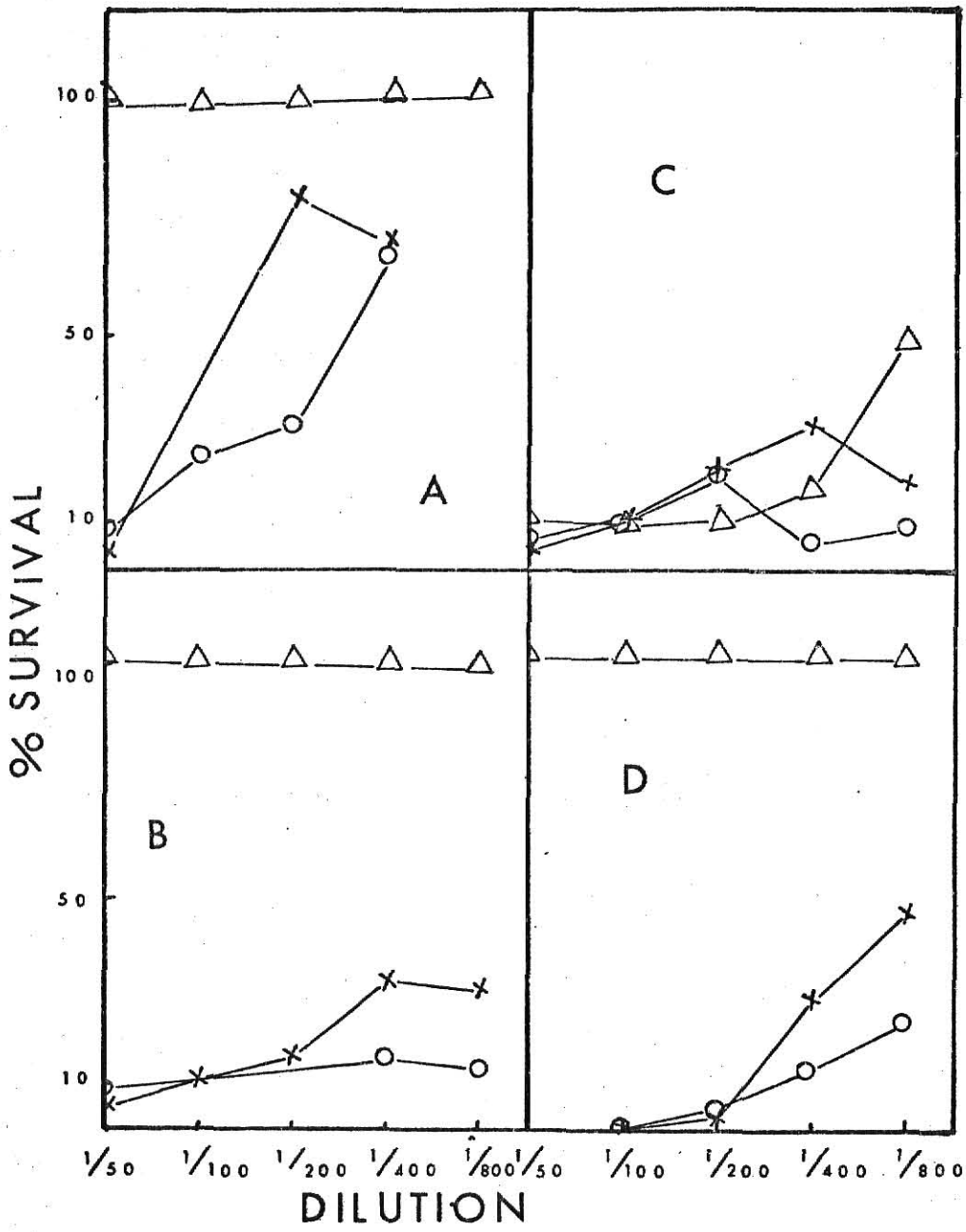

NADL
SINGER
M1
M4
M2
C24V
M3

Figure 6. Cross Neutralization of BVD Virus Isolates in MDBK Cell Cultures Using Guinea Pig and Rabbit Hyperimmune Sera Produced by Crude Antigens. Antisera prepared in guinea pigs against 3 BVD virus strains using crude virus after two subcutaneous injections of 0.25 and 0.5 ml. were cross-reacted in a constant-virus variable-serum neutralization test. Equal volume of antibody dilution (1 1/2 ml.) was mixed with equal volume of virus containing $1.0 \times 10^4 - 4.5 \times 10^4$ PFU/ml. and incubated at 37° C. Serum virus mixture was diluted and plaque assayed. Similarly one rabbit which was hyperimmunized with NADL virus was boosted and its serum was cross-neutralized by the three viruses. Percentage survival was plotted against the antibody dilutions. Symbols: 0———0, NADL virus, Δ—Δ, C24V virus, X———X, Singer virus, A, anti-NADL in guinea pig, B, anti-Singer in guinea pig, C, anti-C24V in guinea pig, D, anti-NADL in rabbit.



A=>NADL; B=>SINGER; C=>$C_{24}V$[G. PIG SERA]
D=>NADL[RABBIT SERUM]

SECTION VI

DISCUSSION

DISCUSSION

The antigenic relationships among BVD virus isolates have been studied by many investigators (Fernelius et al., 1971a; Castrucci et al., 1968a, b and Castrucci et al., 1975).

Field cases of BVD after inoculation with vaccine have been reported by several investigators (Clark, 1968; McKercher et al., 1968; Peacock, 1968; Peter et al., 1967; and Rosner, 1968). It was suspected that vaccine may not protect all vaccinated animals. This may possibly be due to antigenic differences among virus strains used for vaccine production. Significant serological differences among BVD virus isolates as demonstrated by their neutralization indices are not adequate for their classification into distinct serological groups (Corthier and Aynaud, 1973). This classification would require a detailed comparative study of BVD virus isolates by cross serological reactions in order to explore the extent of their antigenic relationships.

Animals inoculated with BVD virus isolates serologically unrelated on the basis of cross neutralization tests demonstrated cross protection when challenged 38 days after vaccination (Castrucci et al., 1975). However, cross protection experiments were not carried out for longer periods to test the duration of immunity. It is evident that many strains of BVD virus exist and detailed studies on the antigenic relationship among these strains is lacking. Antigenic differences of significant

nature may possibly change the immunological properties which may require serious considerations for vaccine production. Consequently, an attempt was made to study the antigenic relationships among three prototype viruses and four field isolates (Minnesota) by cross serum neutralization tests after their preparation in MDBK cells.

BVD Virus Plaque Characteristics: BVD virus plaques produced on confluent monolayers of MDBK cell cultures were most distinct 5-6 days post virus inoculation (Figure 1). Gratzek et al., 1967 showed that BVD virus isolates NADL and C24V produced plaques in calf testicle cultures 3-7 days after infection. They demonstrated that BVD C24V as a vaccinal strain produced larger plaques than the low passage strain NADL and this criteria could differentiate vaccinal strains from field strains in suspected cases of postvaccinal epizootics. In this study BVD-NADL always produced larger plaques than C24V (Table 1, Figure 1). Differences in morphology and plaque size of BVD virus may be due to a certain degree of genetic variation. Dulbecco and Vogt (1954a) reported such differences in RNA viruses, especially togaviruses. The variation in the plaque size may be attributed to irregularities in packaging of viral subunits and a heterogeneous density distribution profile may be observed (Figure 2). Durand and Eisenstark (1962) and Minocha and Consigli (1967) reported similar studies in Newcastle Disease Virus (NDV).

BVD Virus Concentration and Purification: Several procedures have been employed for the concentration and purification of BVD viruses (Scott et al., 1976; Maess and Reczko, 1970; Richie and Fernelius, 1969; Horzinek et al., 1971; Parks et al., 1972; Castrucci et al., 1967; Hafez et al., 1972). These procedures have given a variety of results with either greater losses or higher recovery levels of virus infectivity.

Using 15-30% sucrose gradient and centrifugation at 100,000 g for 3.5 hours Scott et al (1974) were able to obtain BVD virus recovery of 90% with a buoyant density of 1.11 g/cm^3. They obtained a sharp peak of infectious virus indicating that purification procedure had given a homogenous population of undegraded virus particles. Earlier reports by Maess and Reczko (1970) indicated that purification of BVD virus resulted in a loss of 83% of virus infectivity. A heterogenous population with similar loss of virus infective particles (Richie and Fernelius 1969; Horzinek, Maess and Laufs 1971) was also reported using gel filtration and density gradient techniques. Parks et al (1972) were able to purify the virus (NADL) by passing the clarified tissue culture fluid through 20-50% sucrose with the highest recovery of 37%.

Maess and Reczko (1970) using a preformed potassium tartrate gradient (12.5-29% w/v) at 35,000 rpm in SW39 rotor Spinco L50 for two hours were able to purify BVD virus C24V

with 17% recovery of infectivity at a density of 1.115 g/cm^3. The purified virus preparations when examined under an electron microscope displayed an icosahedral shape of virus particles. The method of purification of BVD virus presented in this communication utilized modifications of procedures employed by other investigators.

Enzyme treatment was employed at a low level as compared with the one used by earlier investigators (Castrucci et al., 1967 and Hafez et al., 1972). They used higher trypsin concentration (0.5-1 mg/ml) with one hour incubation as compared to 10-20 µg/ml. trypsin concentration with 30 min. incubation in this study and had low recovery of the virus. There was heterogeneous distribution of BVD virus in a $CsCl_2$ density gradient as determined by the infectivity assay of fractions collected and fraction #14 demonstrating the highest infectivity titer (Figure 2). The distribution profile is synonymous with the studies reported by Horzinek et al., 1971) and Maess et al. (1970). The tailing of the curve is more pronounced towards the bottom of the tube with a steeper slope of the upper boundary. Horzinek et al. (1971) reported that the virus in these fractions contains the least distorted and smallest virus particles.

In all previous purification procedures related to BVD virus no data is available on the virus recovery, protein

content, specific activity and fold purification. Scott et al. (1974) added antisera to aggregate their BVD virus preparations for electron microscopy. In this communication the criteria for BVD virus concentration and purification is shown in Table 2. Total virus decreased from 2.6×10^9 PFU (crude virus) to 1.1×10^9 PFU in the last purification step with 42.3% recovery. Similarly, total protein content decreased from 2.34×10^6 µg to 1.0×10^3 µg. The specific activity (total PFU/total protein) increased three logs in the final step with approximately 1,000 fold purification. These results were variable among BVD virus isolates studied. Assay of purified virus was done immediately after purification. Approximately 1-2 log drop in infectivity occurred when purified virus was frozen and thawed 1-2 times, especially with C24V.

Treatment with RNase, DNase, and trypsin purified the virus from host contaminant (host RNA, DNA and proteins) and resulted in 10 fold purification during that step.

Use of these purified virus preparations in these studies may have two distinct advantages: (1) Response of animals for the production of virus antibody to the specific virus antigen may be enhanced due to low degree of competition by extraneous proteins, and (2) In cross serum neutralization test nonspecific reactions may be avoided.

BVD Virus Density and Infectivity: BVD virus populations studied were highly heterogenous for their density behavior

(Figure 2). Density heterogeneity reflects the chemical and physical complexity of BVD viruses as exemplified by their appearance in the electron microscope and variation in particle size indicates differences in amount of composition of virus structural components (Durand and Eisenstark, 1962). Components derived from host cell material could vary from one virus particle to another, however irregularities in packaging of viral subunits, i.e., nucleoprotein would contribute to density heterogeneity. If the ratios of the protein: nucleic acid components of one particle were not in the same proportion with those of another particle, their density would likewise be different.

Electron Microscopy: Viruses are usually simple and the range of differences in shape and size among viruses is fairly limited. The most important criteria for establishing the relationship between viruses will probably be found to be the gross anatomy of the particles, the absolute weight of the particles and the composition of their nucleic acids. In view of the differences found in composition of the proteins of the related viruses, it would appear that the serological relationships are probably more nearly related to the anatomy than they are to the actual composition of the proteins, i.e. the serological relationships are influenced very much by the way in which the particle subunits are arranged together and less by their actual amino acid composition.

The morphology and classification of BVD virus is not well elucidated. Many investigators have reported a virion structure similar to rubella virus, suggesting its classification with togaviruses. There is still doubt about the morphology and classification of BVD virus although Melnick (1976) has placed BVD virus in the family, pestivirus, a subgenus of the togaviruses. Hermodsson and Dinter (1962) described particles with a diameter of 40 nm. in thin sections. Pritchard (1963) observed spherical particles 35-55 nm. in diameters using chromium shadowing. In the first detailed morphological description the virion was described as 150-250 nm. in size with the structure of myxovirus (Ditchfield and Doane, 1964). An intermediate size of 80 nm. has been reported by Dutta et al. (1964). Other investigators including Hafez et al. (1968) described virions of 22 and 90 nm., depending on the method of preparation. Richie and Fernelius (1969) described three size classes, the largest being 80-100 nm. Maess and Reczko (1970); Horzinek et al. (1971) described sizes of 57 nm. and Scott et al. (1974) reported a particle size of 50-80 nm.

Dulbecco et al. (1973) stated that different methods of virus staining enhance different structural details. The size of the virion, they stated, will be maximal in shadowed preparations which enhance the contrast at the periphery of the particles, it will be smaller in negatively stained

preparations, since the phosphotungstate penetrates the surface details, and it will be even smaller in sections, where the action of the knife tends to collapse the particles. They further added that the values obtained by all these methods are less than the size of the particles in water, since drying, as required for electron microscopic examinations, causes shrinkage by much as 30% in linear dimensions. If the above statements were true, it would be hard to understand the trend in the close agreement in the values of BVD virus particle diameter obtained by most workers, especially when using different methods of preparation (Maess and Reczko (1970); Horzinek et al. (1971).

Purified preparations of NADL, Singer and C24V by potassium tartrate gradient (Table 2) were stained with PTA and examined under the electron microscope. On the morphological grounds it appeared that the envelope had shown considerable variation, being intact in most of NADL virus particles while disappearing or assuming a "dough-like" appearance in preparations of C24V or Singer (Figure 3). The capsomer arrangement and the core symmetry (Figure 3B) would make it likely to suggest that BVD virus particles have displayed a morphological architecture similar to the Sindbis virus described by Horzinek and Mussgay (1969). Although most of the features as described by Caspar and Klug (1962) for isometrical symmetry are lacking it would seem reasonable to describe BVD virus particle as an

icosahedron. Electron microscopic evidence suggests that the virus preparations were sufficiently purified for inoculation into rabbits for the production of antibodies.

Serum Neutralization (SN) Test: Serum neutralization test is the most sensitive of the virus-antibody reactions and has wide application for comparing the antigenic differences between viruses. Prior to the use of purified BVD virus antigens for the production of rabbit hyperimmune sera, preliminary experiments in which crude viruses were used to produce antisera in rabbits and guinea pigs against NADL, Singer and C24V viruses were undertaken . Cross-serum neutralization tests showed that NADL and Singer viruses are closely related and that both are distantly related to C24V (Figure 6). The results of the cross-serum neutralization tests of all seven BVD virus isolates using antisera produced in rabbits with purified virus preparations are presented in Table 4, Figures 4 and 5. The analyses of data revealed that NADL, M_4, Singer and M_1 are closely related and can be suggested to form one group while M_3, C24V and M_2 each forms a separate group. Most of the BVD virus isolates have been found to be related on the basis of serum-neutralization (Gillespie et al. (1961) and Kniazeff et al. (1961) and complement fixation (Gutekunst and Malquist 1964) tests. Castrucci et al. (1967) studied the antigenic relationship of seven BVD virus isolates by serum neutralization test, using

homologous and heterologous antisera prepared in rabbits. When these isolates were compared by Archetti-Horsfall formula (Archetti and Horsfall, 1950) they were grouped into five groups (Castrucci et al., 1968). Four similar studies were carried out on the basis of comparing BVD virus strains. Guneri in 1968 compared ten different BVD viruses isolated in different countries and was able to demonstrate their antigenic relationship. Fernelius et al. (1971) had proposed three prototype strains including NADL, C24V and CG-1220. Corthier and Aynaud (1973) had reported the evidence of significant serological differences between 17 BVD virus isolates including NADL and C24V. They further added that the values of the neutralization indices in their study did not allow them to classify the viruses into distinct serological groups.

In view of the diversity and heterogeneity of BVD virus isolates as compared by their serological reactions, it is logical to believe that the breakdown in immunity in certain animals vaccinated with BVD virus may be due to infection with a virus antigenically unrelated or distantly related. It is also possible that post vaccinal complication in some animals may be related to deficiency in immune system as suggested by McKercher et al. (1968) or Peter et al. (1967).

Dr. D. W. Johnson at Minnesota reported isolation of M_3 from a feeder calf in a herd vaccinated with trivalent vaccine made from IBR, BVD and PI_3 (personal communication).

Similarly, Castrucci et al. (1967, 1968) reported isolation of BVD virus strains antigenically different than the existing virus strains (R values >2). This may possibly indicate mutations occurring in the existing BVD virus strains.

Considering two way antigenic relationships on the basis of R values (Table 5, Figure 5) strong interrelationships exist among the virus isolates that may be categorized as follows:

1. NADL, M_4 and Singer
2. NADL, M_4 and M_1
3. C24V and M_1
4. M_4 and M_2
5. M_3 (not related to any other virus isolates)

It is conceivable a polyvalent vaccine produced using NADL, M_4 and M_1 may be able to protect animals against all the viruses tested except M_3. However, the question of the antigenic relationship with immunogenicity may finally be answered only by cross protection tests in animals.

It was observed that NADL, Singer and M_3 were strong antigens and elicited high a degree of antibody response in rabbits (antibody titer >1/200) to the corresponding antigens (Table 4) while M_1, M_2, M_4 and C24V demonstrated a relatively low degree of antigenic response (antibody titer 1/50). No references are available on the antigenicity of M_1, M_2 and M_4. Hafez et al. (1972a) demonstrated that anti-C24V produced in

rabbits had a titer of 1:45 when reacted with the homologous antigen. Fernelius et al. (1971) had shown that the degree of antigenic response of C24V was approximately 1/4 of the response produced by NADL in calves.

In view of these data it may be suggested that an antigenically weak strain of BVD virus may elicit low antibody titers in rabbits and in calves. It is therefore probable that M_1, M_2 and M_4 which have been observed to give low antibody titers (1/50) in rabbits (Table 4) may also prove to be antigenically weak in calves. Some of these strains do not seem to cover the full spectrum of antigens as investigated by reciprocal cross neutralization test. Thus they may not have good potential for the production of BVD vaccine.

SECTION VII

APPENDIX

MATERIALS AND METHODS

BVD Virus One-Step Growth Curve: BVD viruses, cell culture and media were described previously. The seven BVD virus isolates were each diluted to contain 1.5×10^5 PFU/ml. All viruses were assayed by the plaque technique on MDBK cell cultures.

BVD virus isolate growth curves were monitored in MDBK cell cultures. MDBK cell culture monolayers were grown on 35 x 10 mm. petri dishes (Falcon). Growth medium was aspirated from each dish and virus was inoculated into twenty dishes at a multiplicity of infection of 0.015 PFU per cell. Virus adsorption was allowed to take place for one hour at 37° C in 5% CO_2 humidified incubator. The monolayers were washed twice with PBS and each was fed with one ml. of maintenance medium (MEM) containing 5% horse serum. Two dishes were harvested immediately representing zero-hour sample for each virus. Two dishes from virus inoculated sets were removed at the following intervals: 4, 8, 12, 16, 20, 24, 48, 72 and 96 hours post-inoculation and harvested. The cytopathic effects were observed daily for each sample taken. Undetached cell monolayers for each sample were removed by a rubber policeman, pooled and briefly sonicated and centrifuged at 3,000 g for 10 minutes to remove cell debris. All samples were stored at -70° C before they were plaque assayed as mentioned.

RESULTS

Propagation of BVD Virus: NADL, Singer and C24V viruses had been passaged routinely in MDBK cells. Minnesota field virus isolates M_1, M_2, M_3 and M_4 were passaged three times before they were used for stock virus production. Table 9 presents PFU titers of Minnesota field strains at successive passages. Approximately 1-2 log increase in virus titers (PFU/ml) occurred after third passage in MDBK cells. The viruses were used in the study at either third or fourth passage level.

One-Step Growth Curve: The infectivity titers of seven BVD virus isolates are shown in Table 10 and Figure 7. The adsorption of viruses on cell cultures was not identical, but not significantly different, as indicated by the PFU titers one hour after inoculation of the virus (0 time). During 4-8 hours after inoculation no recoverable virus was demonstrated except in M_3 and M_4 which showed 5-10 PFU/ml. Virus synthesis was first detected at the 12th hour and continued to increase to reach maximum at 96 hours post infection except C24V which showed maximum virus production (8.2×10^6-6.4×10^7) at 48-72 hours. Virus titers observed were: M_4 (3.0×10^7 PFU/ml), Singer (2.6×10^7 PFU/ml), NADL (1.3×10^7 PFU/ml) and M_3 (2.2×10^6 PFU/ml). M_1 and M_2 viruses had minimum infectivity titers (5.1×10^5 and

5.0×10^5 PFU/ml, respectively). The data in Table 10 was graphed and is presented in Figure 7.

DISCUSSION

Propagation of BVD Virus: The propagation of BVD virus in susceptible cells had resulted in an increase in the virus titer by approximately two logs (Table 9) at 3rd passage level. This was similar to earlier reports (Lee and Gillespie (1957), Marcus et al. (1968). Lee and Gillespie (1957) were able to propagate strain 1 virus isolated in New York State in embryonic bovine skin-muscle with titers ranging from 10^5-10^7 PFU/ml. Marcus et al. (1968) were able to adapt NADL strain in MDBK cell after carrying it for six passages in bovine testicle cell cultures, with average titer of 3.3×10^5 PFU/ml. The adsorption time used in this study(1 1/2-2 hours) was similar to that used by Marcus et al. (1968) and Gillespie et al. (1963). It would seem possible that BVD virus would require at least 1 1/2-2 hours for adsorption before satisfactory results could be obtained. Considerable changes in BVD viruses could be induced by their passage into cell cultures. Fernelius et al. (1969) were able to change BVD viruses by swine kidney cell passages. They reported that perhaps some of the strains of BVD virus have originated by swine passage in nature. Beckenhauer et al. (1961) theorized that BVD virus could possibly be passed through swine and back

into cattle again.

One-Step Growth Curve of BVD Virus: The data on the growth curve of BVD virus isolates (Table 10 and Figure 7) were in close agreement with earlier reports of most research workers (Lee and Gillespie (1975); Gillespie (1960); Gillespie et al. (1963) Mills and Luginbuhl (1955); Fernelius et al. (1969); Schiff and Storz (1973); Castrucci et al. (1968a); Gillette (1965) and Singh (1969). The synthesis of infectious BVD virus in MDBK cell cultures began after 8-12 hours post infection. Maximum virus synthesis was observed at 72-96 hours. Small amounts of M_3 and M_4 were detected 4-8 hours after infection which may have been due to the elution of adsorbed virus particles from cell surface. The virus could not be recovered after sonication 4-8 hours after infection in all BVD virus isolates. Cytopathic changes were observed at 24-48 hours and were characterized by granulation and vacuolation of the cytoplasm and as described by Singh (1969). There was no significant difference in the CPE pattern produced by these viruses when grown on MDBK cell cultures except that C24V produced extensive cell damage while M_2 CPE progressed rather slowly. Fernelius et al. (1969) had shown that BVD virus strains have high, intermediate and low cytopathogenicity. Identification of noncytopathogenic (NCP) strains had become possible without the use of natural host when Gillespie and coworkers (1962) employed the plaque

inhibition assay method for detecting NCP strains of BVD virus. The total virus per ml. produced during replication cycle varied from 5.0×10^5 for M_2 to 3.0×10^7 PFU/ml. for M_4 (Table 10, Figure 7). There is a decrease in PFU titer of C24V at 96 hours post infection, possibly due to severe damage to the cells which become detached from the surface with a consequent loss in PFU at 37° C.

TABLE 9. PASSAGE OF BVD VIRUS ISOLATES IN MDBK CELL CULTURES

VIRUS	PASSAGE NUMBER AND TITER (PFU/ML)		
	ORIGINAL VIRUS	1ST PASSAGE	3RD PASSAGE
M_1	6.5×10^4	3.0×10^5	3.1×10^5
M_2	3.5×10^4	1.0×10^5	1.5×10^5
M_3	6.8×10^4	8.0×10^5	2.6×10^6
M_4	1.9×10^5	2.3×10^7	3.8×10^7

TABLE 10. ONE-STEP GROWTH CURVE OF BVD VIRUS ISOLATES GROWN ON MDBK CELL CULTURES

VIRUS	TITER (PFU/ML) AND DIFFERENT HOURS AFTER INFECTION									
	0	4	8	12	16	20	24	48	72	96
NADL	2.5x10′	0	0	7. x10′	2. $x10^3$	$4.25x10^3$	$2.23x10^4$	$2.5x10^5$	$1.5x10^6$	$1.3x10^7$
$C_{24}V$	1.5x10′	0	0	6.5x10′	8. $x10^3$	$3.2x10^4$	$1.2x10^5$	$8.2x10^6$	$6.4x10^7$	$3.0x10^6$
SINGER	5. x10′	0	0	$3.0x10^2$	$1.2x10^4$	$1.4x10^4$	$1.8x10^4$	$1.0x10^6$	$1.4x10^5$	$2.6x10^7$
M_1	1.0x10′	0	0	2.0x10′	2.5x10′	$2.0x10^2$	3. $x10^3$	$6.3x10^3$	$1.5x10^4$	$5.1x10^5$
M_2	1.5x10′	0	0	2.5x10′	3.5x10′	7.0x10′	$6.5x10^2$	$1.3x10^3$	$4.5x10^3$	5. $x10^5$
M_3	8.25x10′	5	5x10′	$2.2x10^4$	$1.9x10^4$	$9.8x10^3$	$5.0x10^4$	$5.3x10^4$	$6.2x10^5$	$2.2x10^6$
M_4	6.8x10′	5	5	$7.5x10^2$	$1.0x10^4$	$1.3x10^5$	4. $x10^4$	$1.8x10^6$	$5.0x10^5$	3. $x10^7$

Figure 7. Growth Curve of BVD Virus Isolates. BVD viruses were inoculated into MDBK cell monolayers with an input of 0.01-0.035 PFU/cell. Virus adsorption was for one hour at 37° C in 5% CO_2 humidified incubator. Monolayers were washed twice with PBS and each dish was fed with maintenance medium (MEM + 5% horse serum). Two dishes were harvested immediately after washing representing 0-hour sample for each virus. Dishes from inoculated virus sets were harvested at the time shown, pooled, sonicated and stored at -70° C before they were plaque assayed. Logs of virus titers (PFU/ml) were plotted against time. Symbols: 0, NADL; △, C24V; X, Singer; □, M_1; ●, M_2; ▲, M_3; ■, M_4.

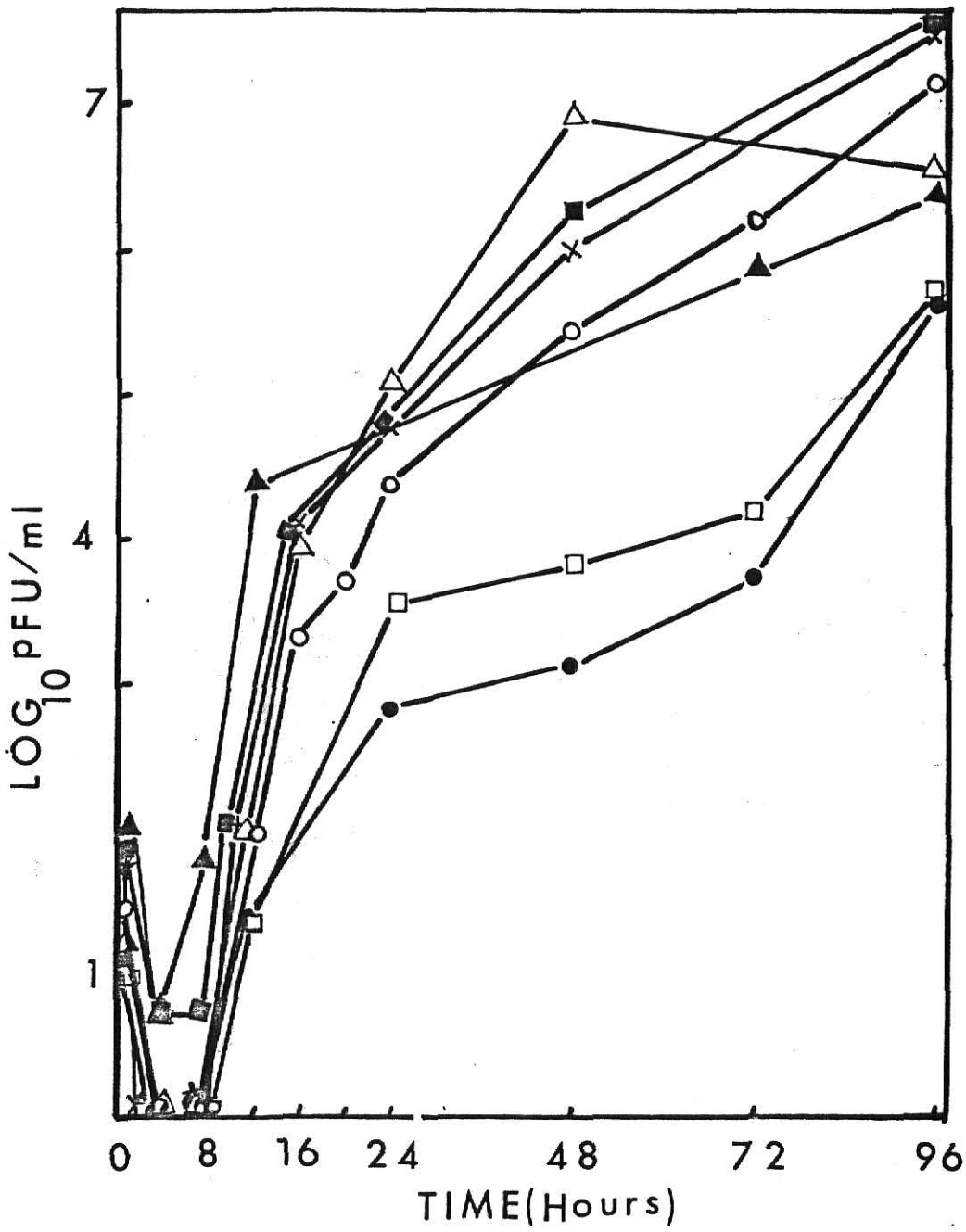

7
4
1
LOG10 PFU/ml
0
8
16
24
48
72
96
TIME(Hours)

FIGURE 8. PROTOCOL OF PURIFICATION OF BOVINE VIRAL DIARRHEA VIRUS.

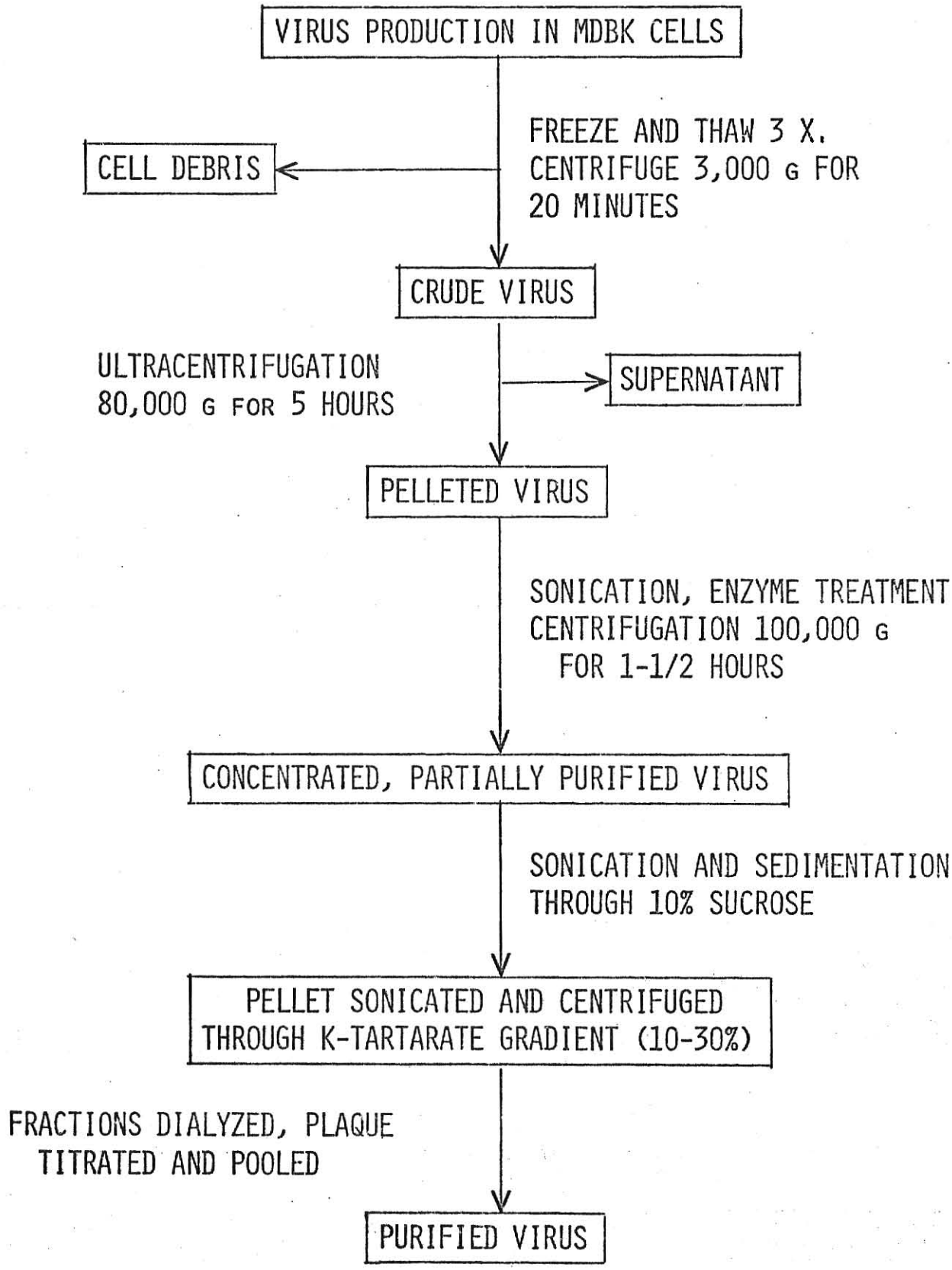
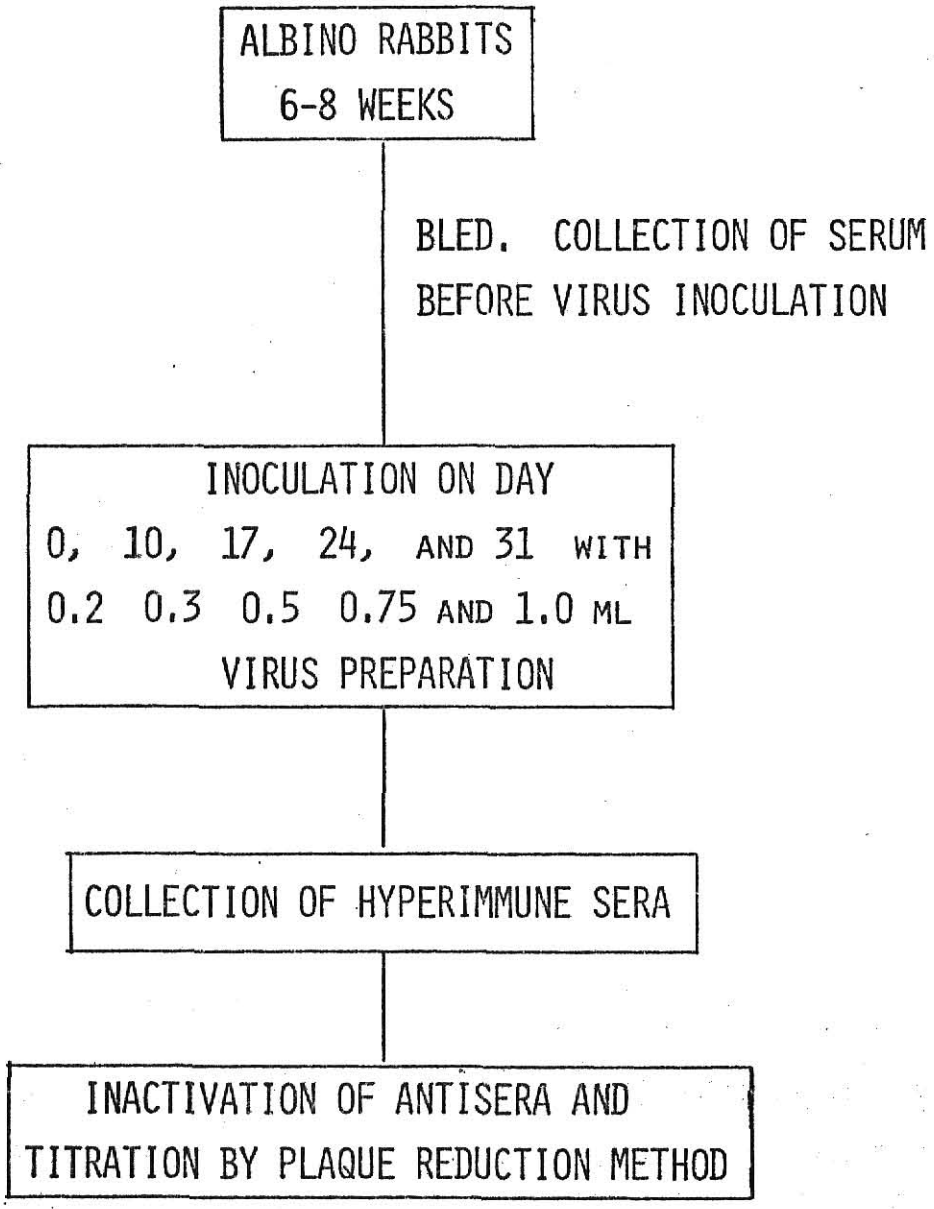

VIRUS PRODUCTION IN MDBK CELLS
FREEZE AND THAW 3 X.
CENTRIFUGE 3,000 G FOR
20 MINUTES
CELL DEBRIS
CRUDE VIRUS
ULTRACENTRIFUGATION
80,000 G FOR 5 HOURS
SUPERNATANT
PELLETED VIRUS
SONICATION, ENZYME TREATMENT
CENTRIFUGATION 100,000 G
FOR 1-1/2 HOURS
CONCENTRATED, PARTIALLY PURIFIED VIRUS
SONICATION AND SEDIMENTATION
THROUGH 10% SUCROSE
PELLET SONICATED AND CENTRIFUGED
THROUGH K-TARTARATE GRADIENT (10-30%)
FRACTIONS DIALYZED, PLAQUE
TITRATED AND POOLED
PURIFIED VIRUS

FIGURE 9. PRODUCTION OF HYPERIMMUNE SERA TO BVD VIRUS.


ALBINO RABBITS
6-8 WEEKS
BLED. COLLECTION OF SERUM
BEFORE VIRUS INOCULATION
INOCULATION ON DAY
0, 10, 17, 24, AND 31 WITH
0.2 0.3 0.5 0.75 AND 1.0 ML
VIRUS PREPARATION
COLLECTION OF HYPERIMMUNE SERA
INACTIVATION OF ANTISERA AND
TITRATION BY PLAQUE REDUCTION METHOD

SECTION VIII

REAGENTS AND SUPPLIERS' ADDRESSES

REAGENTS

Agar

2% purified agar in deionized water

Steam to dissolve agar. Use 50% agar - 50% (2X MEM) for plaquing.

Antibiotics

Penicillin = 1,000,000 units

Streptomycin = 1 g

Kanamycin = 50 mg

dissolved in 100 ml. of sterile deionized water. One ml. of PSK stock solution is added to give final concentrations of: 100 units penicillin, 0.1 g streptomycin and 0.5 mg Kanamycin per ml. of media.

Neutral Red

For use in plaquing as a 0.015% solution. Powder is added to deionized water and dissolved in a 37° C water bath. Whatman #2 filter paper is used for filtration. Sterilize in autoclave and store in refrigerator, protected from light.

1.5 ml. stock per 100 ml. agar and media is used.

Phosphate-Buffered Saline (PBS)

Solution 1

$Na_2HPO_4 \cdot 7H_2O$ 134 g/l deionized water

Solution 2

$NaH_2PO_4 \cdot H_2O$ 69.1 g/l deionized water

Buffer stock

$Na_2HPO_4 \cdot 7H_2O$	386 ml.	Solution 1
$NaH_2PO_4 \cdot 7H_2O$	114 ml.	Solution 2
Deionized water	500 ml.	
	pH 7.15	

PBS: 80 ml. buffer stock
17 g NaCl
1920 ml. deionized water
pH 7.2
Autoclave at 15 lbs. for 15 minutes.

Trypsin

1 g/400 ml. trypsin in PBS made and frozen for use.

Working solution	20 ml.	0.25% trypsin
	30 ml.	PBS
	0.5 ml.	10% EDTA

DNase

2 mg/ml.stock

RNase

2 mg/ml.stock

Sucrose - RNase free

For gradient preparation. Made to appropriate concentration, dissolved and autoclaved 10 minutes at 15 lbs.

Potassium tartrate

For gradient preparation. Made to appropriate concentration, dissolved and autoclaved 10 minutes at 15 lbs.

TEN buffer: (0.01 M tris, 0.001 M EDTA, 0.1M NaCl, adjust pH with HCl to 7.4 and autoclave 10 minutes at 15 lbs.

TRIS-saline: (0.01 M tris, 0.1 M NaCl adjust pH to 7.2 with HCl and autoclave 10 minutes at 15 lbs.

Glycerol:

For gradient preparation. Made to appropriate concentration in TEN buffer and autoclaved 10 minutes at 15 lbs.

Dimethyl sulfoxide $(CH_3)_2\ SO_4$:

For freezing cells. Made to appropriate concentration (10%) and autoclaved 10 minutes at 15 lbs.

REAGENTS AND SUPPLIERS' ADDRESSES

Agar, Purified	BBL Cockeysville, Maryland
Bovine serum albumin	Sigma St. Louis, Missouri
DNase (Pancreatic) RNase	Sigma Chemical Co. Sigma Chemical Co. St. Louis, Missouri
EDTA	Mallinckrodt Chemical Co. St. Louis, Missouri
Horse Serum	Kansas City Biologicals Kansas City, Kansas
Kanamycin (Kantrex)	Bristol Laboratories Syracuse, New York
Minimum Essential Medium Eagles (MEM)	Grand Island Biological Co. New York
Penicillin G	Squibb & Sons New York
Streptomycin Sulfate	Lilly Corporation
Trypsin (crystalline)	Nutritional Biochemical Co. Cleveland, Ohio
Sucrose-RNase free	Schwarz Mann Orangeburg, New York
Potassium tartrate	Fisher Scientific Fairlawn, New Jersey
Glycerol	Mallinckrodt Chemical Works St. Louis, Missouri
Dimethyl sulfoxide	Mallinckrodt Chemical Works St. Louis, Missouri

SECTION IX

REFERENCES

Andrews, C. H. and Piereira, H. G. (1972). Viruses of vertebrates. Bailies Tindall. London. pp. 266-268.

Archetti, I. and Horsfall, F. L., Jr. (1950). Persistent antigenic variation of influenza A viruses after incomplete neutralization in ovo with heterologous immune serum. J. Exp. Med. 92:441-462.

Baker, J. A., Gillespie, J. H., Sheffy, B. E. and Marshall, V. (1958). Simultaneous immunization of cattle against leptospirosis, virus diarrhea and IBR. Cornell Vet. 48:207-213.

Baker, J. A., York, C. J., Gillespie, J. H. and Mitchell, G. B. (1954). Virus diarrhea in cattle. Am. J. Vet. Res. 15:525-531.

Beckenhauer, W. H., Brown, A. L., Lidolph, A. A. and Norden, C. J. (1961). Immunization of swine against hog cholera with bovine enterovirus. Vet. Med. 56:108-112.

Bittle, J. L. and York, C. J. (1968). Combination vaccines for control of bovine respiratory diseases. J.A.V.M.A. 152:889-893.

Blood, D. C., Hutchins, D. R., Jubb, K. V., Whittem, J. H. and Little Johns, I. (1957). "Mucosal disease of cattle in Australia." Australian Vet. J. 33:75-76.

Borgen, H. C. (1963). Mucosal disease in Denmark II relation of viral diarrhea to the spontaneous and experimental disease. Nord. Vet. Med. 15:409-417.

Braun, R. K., Osburn, B. L. and Kendrick, J. W. (1973). Immunologic response of bovine fetus to bovine viral diarrhea virus. Am. J. Vet. Res. 34 (9), 1127-1132.

Bruner, D. W. and Gillespie, J. H. (1973). Hagan's infectious diseases of domestic animals. Cornell University Press. Ithaca, New York: 968-975.

Burki, F. (1965). Bovine virus diarrhea. Quantitative verteilung des Erregers und Isolicrchancen. Path. Microbiol. (Basel) 28:158-166 (cited in Castrucci et al., 1968b).

Cancellotti, F. and others. (1972). Usodi cellule di milza de ecto bovine per L isolamento el' identificazione mediante immunofluorescence di un cerpo di vires della malattia della mucose-diarrhea da viries. Arch. Vet. Halino 23 (6), 435-444. (English summary).

Carlson, R. G., Pritchard, W. R. and Doyle, L. P. (1957). The pathology of virus diarrhea of cattle in Indiana. Am. J. Vet. Res. 18:560-568.

Caspar, D. L. D. and Klug, A. (1962). Physical principles in the construction of regular viruses. Cold Spring Harbour Symp. Quant. Biol. 27:1-24.

Castrucci, G., Avellini, G., Cilli, V. Pedini, B., McKercher, D. G. and Valente, C. (1975). A study of immunologic relationships among serologically heterologous strains of bovine viral diarrhea virus by cross-immunity tests. Cornell Vet. 65 (I): 65-72.

Castrucci, G., Cilli, V. and Galiardi, G. (1968a). Bovine virus diarrhea in Italy. Archiv. fur die gesamte virusforsch. ung. 24:48-64.

Castrucci, G., Avellini, G., Cilli, V., Morettini, B., Bellachioma, F. (1968b). Further studies on bovine viral diarrhea in Italy. Characteristics of strains isolated in Umbria and comparative studies with other strains of Italian or American origin. Boll. lst. sieroter. Milan. 47:341-351.

Chennekatu, P. R., Tyler, D. E. E., Ramsey, F. K. (1967). Characteristics of a condition following vaccination with bovine virus diarrhea vaccine. J.A.V.M.A. Vol. 150 #I:46-52.

Childs, J. (1946). X-disease of cattle-Saskatchewan. Cam. J. Comp. Med. Vet. Sci. 10:316-319.

Clark, F. R. (1968). Immunologic aspects of mucosal disease. Med. Vet. Prac. 49:38-43.

Coggins, L., Gillespie, J. H., Robson, D. S., Thomson, J. D., Phillips, W. V., Wagner, W. C. and Baker, J. A. (1961). Attenuation of virus diarrhea virus (strain Oregon C24V) for vaccine purposes. Cornell Vet. 51:539-554.

Corthier, G. and Aynaud, J. M. (1973). Madadie des moqueuses: Mise en evidemce de vioviation antigeniques entre diverses souches. A luide de la fechuique de seroneutralixation en culture cellulaire. (English summary).

Darbyshire, J. H. (1962). Agar gel diffusion studies with a mucosal disease of cattle. II. A serological relationship between mucosal disease and swine fever. Research Vet. Sci. 3:123-128.

Darbyshire, J. H. (1963). The isolation, separation and identification of two viruses from a case of bovine mucosal disease. J. Comp. Path. 73:309-318.

Diderholm, D. and Dinter, A. (1966). Infectious RNA derived from bovine virus diarrhea virus. ZGL. Bakt., I. Abt. Orig. 201:270-272.

Dinter, Z. and Borgen, H. C. (1964). Bovine virus diarrhea (mucosal disease) virus in Sweden and Danish cattle. Nord. Vet. Med. 16:384-389.

Ditchfield, J. and Doane, F. W. (1964). The properties and classification of bovine virus diarrhea virus. Can. J. Camp. Med. 28:148-152.

Dulbecco, R. (1952). Production of plaques in monolayer tissue culture by single particles of an animal virus. Proc. Natl. Acad. Sci. U.S. 38:747-752.

Dulbecco, R. and Ginsberg, H. S. (1973). The nature of viruses in Microbiology, 2nd edition, by Davis/Dulbecco/Eisen/Ginsberg/Wood. pp. 1007-1085.

Dulbecco, R. and Vogt, M. (1955). Biological properties of poliomyelitis viruses as studied by the plaque technique. Ann. New York Acad. Sci. 61:790.

Durand, D. P. and Eisenstark, A. (1962). Influence of host cell type on certain properties of Newcastle disease virus in tissue culture. Am. J. Vet. Res. 23:338-342.

Dutta, S. K., Shope, R. E. and Pomeroy, B. S. (1964). Electron microscopic structure of bovine virus diarrhea virus. Bact. Proc. 64:III.

Fernelius, A. L. (1964). Noncytopathogenic bovine viral diarrhea viruses detected and titrated by immuno-fluorescense. Can. J. Comp. Med. and Vet. Sci. 38: 121-126.

Fernelius, A. L. (1967). Characterization of bovine viral diarrhea viruses by their interactions with susceptible host cells. Ph.D. thesis, Iowa State University.

Fernelius, A. L. (1968). Characterization of bovine viral diarrhea viruses. I. Determination of buoyant density: Arch. Ges. Virusforsch. 25:211-218.

Fernelius, A. L. and Lambert, G. (1969a). Detection of bovine viral diarrhea virus and antigen in tissues of experimentally infected calves by cell inoculation and fluorescent antibody techniques. Am. J. Vet. Res. 30 (9), 1551-1559.

Fernelius, A. L. and Packer, R. A. (1969b). Serological reactions of bovine diarrhea viruses with anticellular and antivirus sera produced in rabbits. J. Gen. Virol. 5:243-249.

Fernelius, A. L., Lambert, G. and Booth, G. D. (1971a). Bovine viral diarrhea virus-host cell interaction: serotypes and their relationship to biotypes by cross neutralization. Am. J. Vet. Res. 23 (2): 229-238.

Fernelius, A. L., Classick, L. G. and Smith, R. L. (1971b). Evaluation of a soluble antigen vaccine prepared from bovine viral diarrhea-mucosal disease virus-infected cell cultures. Am. J. Vet. Res. 32 (12): 1963-1979.

Fernelius, A. L. and Ritchie, A. E. (1966). Mixed infection or contaminations of bovine viral diarrhea virus with infectious rhinotracheities virus. Am. J. Vet. Res. 27:241-248.

Fernelius, A. L. and Velicer, L. F. (1968). Characterization of bovine viral diarrhea viruses. III. Partial purification by agar filtration. Arch. Soc. Virusforsh. 25:227-234.

Frost, J. W. and Leiss, B. (1973). Separation of structural proteins of bovine viral diarrhea virus after degradation by different splitting techniques. Arch. Gesamte. Virusforsch. 42:297-299.

George, T. D. st. (1971). A survey of sheep throughout Australia for antibody to parainfluenza type 3 virus and to mucosal disease virus. Aust. Vet. J. 47:428-433.

Gillespie, J. H., Baker, J. S. (1959). Studies on virus diarrhea. Cornell Vet. 49:439-443.

Gillespie, J. H., Baker, J. A. and McEntee, K. (1960). Cytopathogenic strain of virus diarrhea virus. Cornell Vet. 50:73-79.

Gillespie, J., Bartholomew, R., Thomson, P. and McEntee, K. (1967). The isolation of noncytopathic virus diarrhea virus from two aborted bovine fetuses. Cornell Vet. 57:564-571.

Gillespie, J. H., Coggins, L., Thomson, J. and Baker, J. A. (1961). Comparison by neutralization tests of strains of virus isolated from virus diarrhea and mucosal disease. Cornell Vet. 51:155-159.

Gillespie, J. H., Madin, S. H. and Darby, N. (1962). Cellular resistance in tissue culture induced by noncytopathogenic strains to a cytopathogenic strain of virus diarrhea virus of cattle. Proc. Soc. Exptl. Biol. Med. 110:248-250.

Gillespie, J. H., Madin, S. H. and Darby, N. B. (1963). Studies on virus diarrhea virus of cattle, with special reference to its growth in tissue culture and its susceptibility. Cornell Vet. 53:276-282.

Gillette, K. G. (1965). Characterization studies of two strains of bovine viral diarrhea virus. Diss. Abstr. 27B:363-364.

Gratzek, J. B. (1962). Characteristics of two bovine viral diarrhea agents. Dissertation Abs. 22 (8-9), 2926.

Gratzek, J. B., Peter, C. P. and Buening, G. M. (1967). Plaque characterization of four classes of bovine viruses. Am. J. Vet. Res. 28:641-646.

Guneri, S. (1968). Vergleichende Serologische Untersuchungen uber Beziehungen Zwischen Verschiedenen Mucosal Disease (Virus-diarrhoea) Virusstammen. Inaug. Diss. Tierarztl. Fak. Munchen. p. 32 (cited in Castrucci et al., 1975).

Gutekunst, D. E. (1968). Comment on vaccination for bovine viral diarrhea-mucosal disease. J.A.V.M.A. 152:889-893.

Gutekunst, D. E. and Malquist, W. A. (1964). Complement-fixing and neutralizing antibodies response to bovine viral diarrhea and hog cholera antigens. Cand. J. Comp. Med. and Vet. Sci. 28:19-23.

Gutekunst, D. E. and Malquist, W. A. (1965). Soluble antigen of bovine viral diarrhea virus: I. Intracellular synthesis, separation and characterization. Arch. Soc. Virusforsh. 15:159-168.

Hafez, S. M. and Liess, B. (1972a). Studies on bovine viral diarrhea-mucosal disease virus. I. Cultural behavior and antigenic relationship of some strains. Acta Virol. 16: 388-398.

Hafez, S. M. and Leiss, B. (1972b). Studies on bovine viral diarrhea-mucosal disease virus. II. Stability and some physiochemical properties. Acta Virol. 16:399-408.

Hafez, S. M., Petzoldt, K. and Reczko, E. (1968). Morphology of bovine viral diarrhea virus. Acta Virol. 12:471-472.

Hedstrom, H. and Isaksson, A. (1951). Epizootic enteritis in cattle in Sweden. Cornell Vet. 41:251-253.

Hermodsson, S. and Dinter, S. (1962). Properties of bovine virus diarrhea virus. Nature. 194:893-894.

Hore, D. E., Smith, H. V., Snowdon, W. A. and Uren, J. (1973). An investigation of viral and bacterial agents associated with ovine perinatal mortality. Australian Vet. J. 49: 190-195.

Horzinek, M., Maess, J. and Laufs, R. (1971). Studies on the substructure of toga-viruses. II. Analysis of equine arteritis, rubella, bovine viral diarrhea and hog cholera viruses. Arch. Ges. Virusforsch 33:308-318.

Horzinek, M. and Mussgay, M. (1969). Studies on the nucleocapsid structure of a group A arbovirus. J. of Virology 4:514-520.

Howarth, J. A. (1972). A manual of infectious diseases. Library Univ. of Cal., Davis. 191-194.

Huck, R. A. (1957). Mucosal disease complex. J. Comp. Pathol. Therap. 67:267-276.

Ifft, J. B., Voet, D. H. and Vinegrad, J. (1961). The determination of density distributions and density gradients in binary solutions at equilibrium in the centrifuge. J. of Physical Chemistry 65:1138-1145.

Jensen, Rue and Mackey, D. R. (1971). Diseases of feedlot cattle. Lea and Febiger, Philadelphia:6-13.

Khars, R. F. (1968). The relationship of bovine viral diarrhea mucosal disease to abortion in cattle. J.A.V.M.A. 153: 1652-1655.

Khars, R. F. (1971). Differential diagnosis of bovine viral diarrhea-mucosal disease. Am. Vet. Med. Ass. 159 (II), 1383-1386.

Khars, R. F., de lahunta, A., and Scott, F. W. (1970b). Bovine viral diarrhea-mucosal disease, abortion and congenital and cerebellar hypoplasia in a dairy herd. J.A.V.M.A. 156:851-857.

Khars, R. F., Scott, F. W., de lahunta, A. (1970a). Congenital cerebellar hypoplasia and ocular defects in calves following bovine viral diarrhea-mucosal disease infection in pregnant cattle. J.A.V.M.A. 156 (10):1443-1450.

Kniazeff, A. J., Huck, R. A., Jarrett, W. F. H., Pritchard, W. R., Ramsey, F. K., Schipper, I. A., Stober, M., and Liess, B. (1961). Antigenic relationship of some bovine viral diarrhea-mucosal disease viruses from the United States, Great Britain and West Germany. Vet. Rec. 73:768-769.

Kniazeff, A. J. and Pritchard, W. A. (1960). Antigenic relationship in the bovine viral diarrhea mucosal disease complex. Proc. 62nd Ann. Meet. U.S. Livestock Sanitary Assoc. 344-350.

Kniazeff, A. J. and Walker, J. B. (1963). Plaque assay of viral diarrhea virus Oregon C24V (cited by Singh who cited that from W. R. Pritchard in Adv. Vet. Science 8:29).

Lambert, G., Fernelius, A. L. and Cheville, N. F. (1969). Experimental bovine diarrhea in neonatal calves. J.A.V.M.A. 154:181-189.

LaPlaca, M., Portolani, M. and Lamieri, C. (1965). The basis for a classification of bovine enteroviruses. Archiv. fur die gesamte virusforschung. 16-17, 98-115.

Lee, K. M. and Gillespie, J. H. (1957). Propagation of virus diarrhea virus of cattle in tissue culture. Am. J. Vet. Res. 18:952-953.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the folin phenol reagent. Journal of Biological Chemistry 193:265-275.

Lwoff, A. (1962). The thermosensitive critical event of the viral cycle. Cold Spring Harbour Symposia Quant. Biol. 27:159-174.

Madin, S. G. and Darby, N. B. (1958). Established kidney cell lines of normal adult bovine and ovine origin. Proc. Soc. Exptl. Biol. and Med. 98:574-576.

Maess, J. and Reczko, E. (1970). Electron optical studies of bovine viral diarrhea-mucosal disease virus (BVDV). Arch ges virusforsch. 30:39-46.

Malquist, W. A. (1968). Bovine viral diarrhea-mucosal disease: Pathology and applied immunology. J.A.V.M.A. 154:763-768.

Malquist, W. A., Fernelius, A. L. and Gutekunst, D. E. (1965). Interference of bovine viral diarrhea virus by hog cholera virus in swine kidney cell cultures. Am. J. Vet. Res. 26:1916-1927.

Marcus, S. J. and Moll, T. (1968). Adaptation of bovine viral diarrhea virus to the Madin-Darby bovine kidney cell cultures. Am. J. Vet. Res. 29:817-819.

McKercher, D. G., Saito, J. K., Grenshaw, G. L. and Bushnell, R. B. (1968). Complications in cattle following vaccination with a combined bovine viral diarrhea-IBR vaccine. J.A.V.M.A. 152:1621-1624.

Melnick, J. L. (1976). Taxonomy of viruses. Prog. Med. Virol. 22:211-221 (Karger, Basel 1976).

Mengeling, W. L., Gutekunst, D. E., Fernelius, A. L. and Pirtte, E. C. (1963). Demonstration of an antigenic relationship between hog cholera and bovine viral diarrhea viruses by immunofluorescence. Cand. J. Comp. Med. and Vet. Sci. 27:162-164.

Miller, M. F. II. (1974). Particle counting of viruses. In Hayat, M. A. Principles and techniques of electron microscopy. 4:89-128. Van Nostrand Reinhold. N.Y. 21 p.

Mills, J. H. L. and Luginbull, R. E. (1965). Third passage bovine kidney cell cultures. An excellent system for propagating the Oregon, C24V, mucosal disease agent. Cornell Vet. 55:344-354.

Mills, J. H. L. and Luginbull, R. E. (1968). Distribution and persistence of mucosal disease virus in experimentally exposed calves. Am. J. Vet. Res. 29:1367-1395.

Minocha, H. C. and Consigli, R. A. (1967). The concentration of polyoma virus by ammonium sulphate precipitation and purification by density gradient centrifugation: Transactions of Kansas Academy of Science 70:201-209.

Minocha, H. C. and Consigli, R. A. (1968). Concentration and purification of Newcastle disease virus. Am. J. Vet. Res. 29:877-881.

Moennig, V. C. (1971). Untersuchungen uber das sedimentation-suerhatten der Ribonukleinsaure des virus der mucosal-virus-diarrhoe: Ein Beitrag zu seines klassifizierung als mitglied der togavirusgruppe. Thesis, Hanover (cited in Hafez et al., 1972b).

Noice, R. and I. A. Schipper. (1959). Isolation of mucosal disease virus by tissue culture in mixture 199, Morgan, Morton, and Parker. Proc. Soc. Exp. Biol. (N.Y.) 100: 84-87.

Olafson, P., MacCallum, A. D. and Fox, F. H. (1946). An apparently new transmissible disease of cattle. Cornell Vet. 36:205-213.

Olafson, P. and Richard, C. G. (1947). Further observations on the virus diarrhea (new transmissible disease) of cattle. Cornell Vet. 37:104-106.

Parks, J. B., Pritchett, R. F. and Zee, Y. C. (1972). Buoyant density of bovine viral diarrhea virus (365II). Proc. Soc. Exp. Med. 140:595-598.

Peacock, G. V. (1968). Comments on complications that arise after vaccination against bovine respiratory disease. J.A.V.M.A. 152:905-907.

Peter, C. P., Tyler, D. E. and Ramsey, F. K. (1967). Characteristics of a condition following vaccination with bovine virus diarrhea vaccine. J.A.V.M.A. 150: 46-52.

Phillip, J. I. H. (1968). Viral respiratory diseases. Vet. Rec. 82 (10), iv-vii.

Phillips, R. M., Heuschele, W. P. and Todd, J. D. (1975). Evaluation of a bovine viral diarrhea vaccine produced in a porcine kidney cell line. Am. J. Vet. Res. 36: 135-140.

Pritchard, W. R. (1963). The bovine viral diarrhea-mucosal disease complex. Adv. Vet. Sci. 8:1-47.

Pritchard, W. R., Carlson, R. G., Moser, H. E. and Taylor, D. E. B. (1955). Virus diarrhea and mucosal disease. Proc. 59th Ann. Meeting U.S. Livestock Sanitary Assoc: 73-188.

Pritchard, W. R., Taylor, D. B., Moses, H. E. and Doyle, L. P. (1956). Transmissible disease affecting the mucosae of cattle. J. Am. Vet. Med. Assoc. 128:1-5.

Pritchett, R. F. and Zee, Y. V. (1975). Structural proteins of bovine viral diarrhea virus. Am. J. Vet. Res. 36 (12): 1731-1734.

Ramsey, F. K. and Chivers, W. H. (1953). Mucosal disease of cattle. North Am. Veterinarian 34:629-633.

Ritchie, A. E. and Fernelius, A. L. (1969). Characterization of bovine viral diarrhea viruses. V. Morphology of characteristic particles studied by electron microscopy. Arch. Ges Virusforsch. 28:369-389.

Rosner, S. F. (1968). Complications following vaccination in cattle against IBR, bovine viral diarrhea-mucosal disease, and parainfluenza type 3. J.A.V.M.A. 152: 898-902.

Schiff, L. J., Storz, J. and Collier, J. R. (1973). Kinetics of viral adsorption in singly and multiply infected bovine cells. Am. J. Vet. Res. 34 (II):1453-1455.

Scott, E. J., June, D., Almida and Oireilly, K. J. (1974). Characterization of mucosal disease virus as togavirus by electron microscopy. Microbios. 46:79-84.

Sheffy, B. E., Coggins, L. and Baker, J. A. (1962). Relationship between hog cholera virus and virus diarrhea virus of cattle. Proc. Sci. Expt. Biol. Med. 109:349-352.

Siebold, H. R. and Dougherty, E. (1967). Virus diarrhea virus neutralizing substance in bovine and porcine serums. Am. J. Vet. Res. 28:137.

Singh, K. V. (1969a). Intranuclear inclusions in bovine embryo cell cultures infected with bovine virus diarrhea-mucosal disease virus. Vet. Record (84) 230-232.

Singh, K. V. (1969b). A study of bovine virus diarrhea mucosal disease virus by plaque technique. Cand. J. Comp. Med. Vol. 33:207-213.

Snowdon, W. A. and French, E. L. (1968). The bovine mucosal disease-swine fever complex in pigs. Australian Vet. J. 44:179-184.

Snowdon, W. A., Parsonson, I. M. and Brouncsiro, Mary Lou. (1975). The reaction of pregnant ewes to inoculation with mucosal disease virus of bovine origin. J. Comp. Path. 85:241-251.

Tamoglia, T. W. (1968). Laboratory evaluation of bovine respiratory disease vaccines for safety. J.A.V.M.A. 152:847-850.

Tanaka, Y., Unaba, Y., Omari, T. and Matsumoto, M. (1968). Bovine diarrhea virus. III. Physiochemical properties of bovine diarrhea virus. Jap. J. Micro. 12:201-210.

Taylor, G. N., Gustafson, D. P. and Claflin, R. M. (1963). Properties of some viruses of the mucosal-disease virus diarrhea complex. Am. J. of Vet. Res. 24:143-194.

Thomson, R. G. (1974). Pathology and pathogenesis of the common diseases of the respiratory tract of cattle. Cand. J. Comp. Med. 15:249-251.

Thomson, R. G. and Savan, M. (1963). Studies on virus diarrhea and mucosal disease of cattle. Can. J. Comp. Med. Vet. Sci. 27:207-214.

Truitt, R. L. and Sclcheister, L. L. (1973). The replication of bovine viral diarrhea-mucosal disease virus in bovine leukocytes in vitro. Archiv. fur die gesamte virusforsch. ung 42:78-87.

Tyler, D. E. and Ramsey, F. K. (1968). Comparative pathologic, immunologic, and clinical responses produced by selected agents of the bovine mucosal disease-virus diarrhea complex. Am. J. Vet. Res. 26 (113):903-913.

Underdahl, N. R., Grace, O. D. and Hoerlein, A. B. (1957). Cultivation in tissue culture of cytopathogenic agent from bovine mucosal disease. Proc. Soc. Exptl. Biol. Med. 94:795-797.

Walker, R. V. L. and Olafson, P. (1947). Failure of virus diarrhea of cattle to immunize against rinderpest. Cornell Vet. 37:107-111.

Ward, G. M. (1961). Experimental infection of pregnant sheep with bovine viral diarrhea-mucosal disease virus. Cornell Vet. 61:179-191.

Ward, G. H. (1969). Bovine cerebellar hypoplasia apparently caused by BVD-MD virus; a case report. Cornell Vet. 59 (4):570-576.

Watson, D. H., Russell, W. C. and Wildy, P. (1963). Electron microscopic particle counts on herpesvirus using the phosphotungstate negative staining technique. Virology 19:250-260.

Wheat, J. D., McKercher, D. C. and York, C. J. C. (1954). Virus diarrhea in California. Calif. Vet. 7:26-27, 35.

THE ANTIGENIC RELATIONSHIPS AMONG BOVINE VIRAL DIARRHEA VIRUS ISOLATES

by

BASHIR HAMID TEIRAB

B.V.Sc., University of Khartoum, Sudan, 1966

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Infectious Diseases

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1977

ABSTRACT

The antigenic relationship among seven bovine viral diarrhea (BVD) virus strains NADL, C24V, Singer, and four field isolates from Minnesota (M_1, M_2, M_3 and M_4) were studied by constant-virus variable serum cross-neutralization reactions. The viruses were propagated and plaque assayed in Madin-Darby bovine kidney (MDBK) cells.

The virus synthesis in cell cultures was initiated at 12 hours post infection and reached maximum synthesis at 72-96 hours. Mean plaque size diameter of the virus isolates was different and ranged from 1.8 to 4.3 mm. All BVD virus isolates produced plaques with diffusable boundaries except C24V which had sharp clear-cut margin.

The viruses were clarified and concentrated by differential centrifugation. BVD virus was purified 109 fold after enzyme treatment (trypsin, RNase and DNase) with 77% recovery. The comparison of sucrose, potassium tartrate and cesium chloride gradients for further purification demonstrated maximum recovery of the virus (37-42%) in potassium tartrate gradients.

Three BVD virus isolates (NADL, C24V and Singer) were studied by electron microscopy. The nucleocapsid displayed an icosahedron symmetry with a smooth envelope which was 25-39 nm. across. The capsid had a mean diameter of 106-120 nm.

Hyperimmune serum was produced in rabbits using purified virus preparations. NADL, Singer and M_3 elicited the highest antibody response in rabbits (1/200) while C24V, M_1, M_2 and M_4 produced antibody titer ranging from 1/50 to 1/100. Reciprocal cross-neutralization tests were carried out to investigate antigenic relationships among the virus isolates. The results of these tests were subjected to Archetti-Horsfall (1950) formula and computer analysis. A strong two-way antigenic relationship was observed among NADL, Singer and M_4; likewise M_1 was related to NADL, M_4 and C24V. M_2 had two-way relationship only with M_4; and M_3 demonstrated a weak relationship with the viruses tested. Computer analysis of the cross-serological tests among the virus isolates was essentially in accord with the antigenic relationships established using Archetti-Horsfall formula.