

Noncytopathogenic Bovine Viral Diarrhea Viruses Detected and Titrated by Immunofluorescence

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SUMMARY

Noncytopathogenic (NCP) bovine viral diarrhea (BVD) disease agents can be detected and titrated in tissue culture systems by a method employing immunofluorescence. Cytopathogenic (CP) and NCP viruses cross react with fluorescein-conjugated serum globulins produced against either CP and NCP viruses, but the fluorescence is more intense in the homologous system. Serum neutralization titers of sera against both CP and NCP groups were compared for both groups of viruses, and results of cross reactions were in agreement with results from immunofluorescence tests. Results of these two tests were discussed as to possible antigenic groupings of CP and NCP viruses. Use of immunofluorescence as a diagnostic test for BVD and as an alternate method of titrating NCP viruses in tissue culture systems is proposed.

Introduction

Confirmatory diagnoses of bovine viral diarrhea (BVD) disease complexes originally depended upon transmissions of the disease by calf inoculations (1,2). Lee and Gillespie (3) described a method of propagating and maintaining in tissue culture, New York-1, a noncytopathogenic (NCP) strain of BVD virus. This method also required the use of calves to detect and titrate the tissue culture-produced virus because the virus did not produce a cytopathic effect (CPE) on the tissue culture systems employed for viral propagation. Underdahl, et al. (4), reported cultivation in tissue culture of a cytopathogenic (CP) agent from bovine mucosal disease in 1957. Identification of NCP strains became possible without the use of calves when Gillespie and coworkers (5) employed a plaque inhibition assay method for measuring

NCP strains of BVD virus. This assay was based on the principle that tissue culture cells become resistant to a plaque-forming CP strain of BVD virus if the NCP strain had been inoculated at least three days earlier.

The present paper describes a method of detection and titration of NCP strains of BVD viruses by the use of immunofluorescence, and compares this method with serum neutralization and tissue culture titration methods.

Materials and Methods

Virus Cultures. Primary bovine embryonic kidney (BEK) cells were cultured on coverslips in Leighton tubes in media described by Gutekunst and Malmquist (6). Cultures were infected with CP strains: Oregon C24V or NADL-MD, or with NCP strains: New York 1 (NY-1), Indiana 46 (IND-46), Sanders 78 (SAN-78), Merrell 171 (MER-171) or CG-1220. Typical examples of CPE or its absence in BEK cells are shown in Figure 1. Details of isolation source and cell passage are given in Table 1. Several tissue culture passages were necessary to adapt these viruses to tissue culture cells to obtain a suitable virus titer.

Serum conjugates: Antisera against NADL-MD and CG-1220 viruses were prepared in specific pathogen-free (SPF) calves (9). Neutralization titers of these sera were 1:3365 and 1:2880 respectively against the homologous virus. Sera were conjugated with fluorescein isothiocyanate by a method reported earlier (10), with the additional millipore filtration step added as reported by Mengeling et al. (11). Both CP and NCP viruses were stained with these serum conjugates.

Slide Preparation. Details of slide preparations and observations by fluorescence microscopy have previously been described (10) except that NCP viruses were in-

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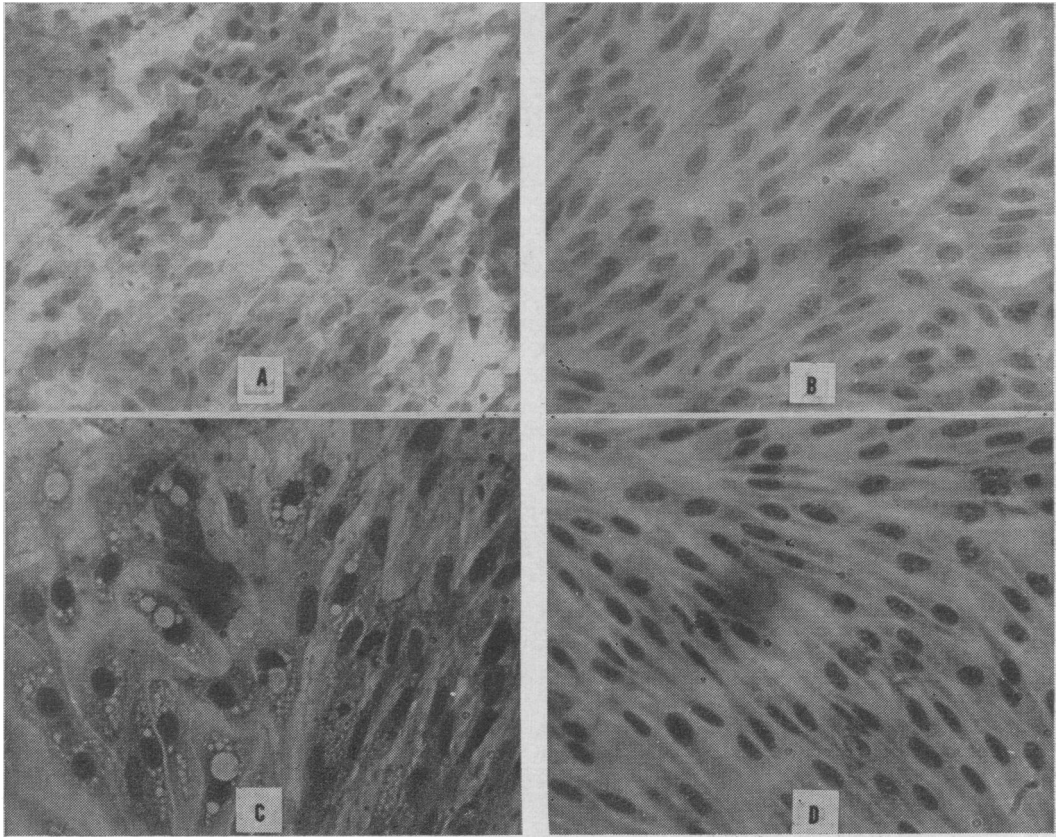


Figure 1: Bovine embryonic kidney cell monolayers 72 hours after viral infection. Stain is Harris' hematoxylin-eosin. A) NADL-MD, a CP virus. B) CG-1220, a NCP virus. C) C24V, a CP virus. D) Noninfected control cells.

TABLE 1. — Cytopathogenic and Noncytopathogenic Strains of VD-MD Viruses Investigated by an Immunofluorescence Method

Virus Designation	Cytopathogenicity for BEK Cells	Isolation Details	Supplier and Subsequent Passages
C24V	CP	Oregon material, isolated in New York by Gillespie, et al. (7)	Kniazeff, Gainesville, Fla. 9 BEK passages
NADL-MD	CP	NADL herd, Ames, Iowa by Gutekunst & Malmquist (6)	Gutekunst & Malmquist 8 BEK passages
NY-1	NCP	New York by Baker et al. (2)	Gillette, Purdue Univ. Calf passage, then 8 BEK passages.
IND-46	NCP	Indiana by Pritchard (8)	Tyler, Iowa State Univ. Calf passage, then 8 BEK passages
SAN-78	NCP	Iowa by Trapp	Tyler, Iowa State Univ. Calf passage, then 8 BEK passages
MER-171	NCP	Purdue group Lafayette, Ind.	Purdue Univ. group, Lafayette, Ind. 8 BEK passages.
CG-1220	NCP	Iowa cattle (9)	Malmquist, NADL 9 BEK passages

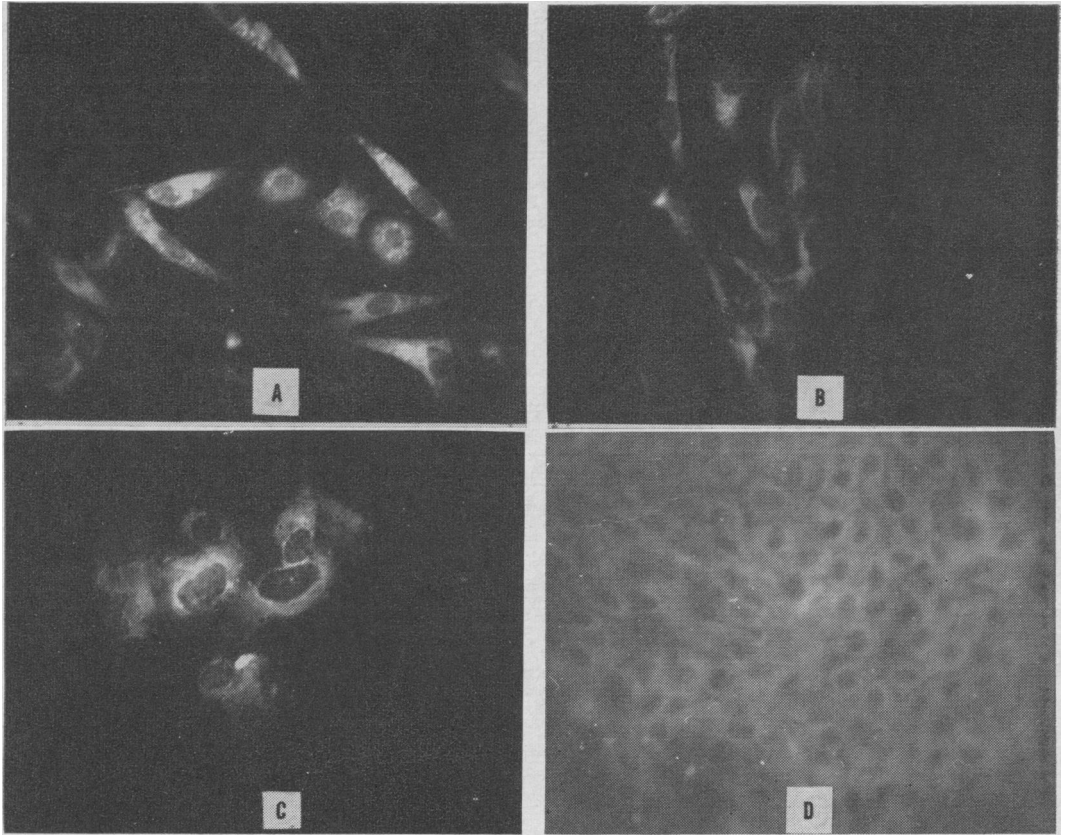


Figure 2: Bovine embryonic kidney cell monolayers 72 hours after viral infection. Stain is fluorescein-conjugated BVD globulins. A) NADL-MD + anti-nadl globulin. B) CG-1220 + anti-cg-1220 globulin. C) C24V + anti-nadl globulin. D) Noninfected control cells + anti-nadl globulin.

cubated in Leighton tube cultures for 72 to 96 hours rather than the 36 to 40 hours incubation time required for optimal fluorescence of CP viruses.

Titration. Serum neutralizations and titrations of CP viruses were conducted as described by Gutekunst and Malmquist (6). Serum neutralizations and titrations of NCP viruses were performed by an interference method described by Gillespie et al. (5) and modified by Gutekunst and Malmquist (9). Both CP and NCP viruses were titrated by an immunofluorescence method consisting of inoculations of BEK cultures, grown on coverslips in Leighton tubes, with ten-fold dilutions of each virus. After incubation, coverslip cultures were washed with pH 7.2 phosphate buffered saline (12), fixed in absolute acetone at -60°C , stained with fluorescein isothiocyanate conjugates and observed for specific fluorescence (13) by fluorescence microscopy.

Results

Detection of Viruses in Experimentally Infected Tissue Cultures. Bovine embryonic kidney cells infected with NCP or with CP viruses could be stained with either fluorescein-conjugated NADL-MD globulin or with CG-1220 globulin (Table 2). These globulins were produced in calves from CP and NCP viruses respectively. Typical examples of fluorescence of infected cells are shown in Figure 2. Results in Table 2 show that immunofluorescence is a tool with which NCP viruses can be identified in tissue culture by the application of fluorescein-conjugated serum globulins from either CP or NCP virus-infected or hyperimmunized animals. The intensity of fluorescence of cells infected with NCP viruses and stained with labeled NCP viral anti-globulin (anti-CG-1220) was brighter than that of NCP virus-infected cells stained with labeled CP viral

TABLE 2. — Immunofluorescence and Serum Neutralization (SN) Titers when Cytopathogenic (CP) and Noncytopathogenic (NCP) Viruses are Reacted with Anti-NADL-MD and Anti-CG-1220 Sera and Serum Globulins

Strain of Virus Reacted		A Fluorescence with Labeled Anti-NADL-MD Globulin	B SN Titer of Anti-CP Viral (NADL-MD) Serum Against 100 TCID ₅₀ of Virus	C Fluorescence with Labeled Anti-CG-1220 Globulin	D SN Titer of Anti-NCP Viral (CG-1220) Serum Against 100 TCID ₅₀ of Virus
CP	(C24V	++++*	120**	+++	218
	(NADL-MD	++++	3365	+++	1289
NCP	(NY-1	++	181	+++	512
	(IND-46	++	63	+++	875
	(SAN-78	++	208	+++	879
	(MER-171	++	120	+++	879
	(CG-1220	++	1450	+++	2880
	(BEK Control Cells	--	0	--	0

*Intensity of fluorescence (13):

- 4+ Maximal fluorescence; brilliant yellow-green.
- 3+ Bright yellow-green fluorescence.
- 2+ Less brilliant, but definitely fluorescence.
- 1+ Fluorescent, but dull.
- No fluorescence.

**Expressed as reciprocals of serum dilutions.

antiglobulin (anti-NADL-MD). Cells infected with CP viruses stained maximally with fluorescein-conjugated anti-globulins produced from either NCP or CP viruses.

Neutralization Tests. Serum neutralization (SN) titers of the NCP antiserum, CG-1220, were higher against the NCP virus strains than were the SN titers of the CP antiserum, NADL-MD, against the NCP virus strains (Table 2). These higher serum neutralization titers are in agreement with the higher degrees of intensity of fluorescence of these NCP virus strains mentioned in the preceding paragraph. An exception to this observation is the low SN titers and the high degree of fluorescence with the CP strain, C24V.

Titration of Viruses by an Immunofluorescence Method. Results in Table 3 show agreement between immunofluorescence and tissue culture infectivity titration methods. Endpoints of infectivity for both CP and NCP viruses correlate well with endpoints of immunofluorescence. Viral titers as TCID₅₀ per ml are given for each virus.

Discussion

Evidence is presented in this paper that BVD viruses which do not produce a CPE in tissue cultures can be identified in tissue cultures by a method employing immunofluorescence. Fluorescein-conjugated serum globulins produced in calves against either a CP strain or against an NCP strain of BVD virus can be used to identify these NCP viral-infected tissue culture cells. It is suggested that a similar immunofluorescence test may be employed to diagnose BVD in infected cattle by either direct examination of infected tissue sections or by examination of fluorescent antibody-stained tissue culture cells inoculated with blood, serum, nasal secretions or other pathological specimens from the diseased cattle. Such diagnostic tests have been described for hog cholera (11,14).

Concentrations of NCP-BVD viruses can also be titrated by immunofluorescence in a given viral culture or other preparation by detection of the endpoint of infection of tissue culture cells by the method shown in Table 3. In this study, to conserve the

TABLE 3. — Comparisons of Titrations of Viruses by Tissue Culture Infectivity (TI) and Immunofluorescence (IF) Methods

Logarithmic Dilution of Virus	CP						NCP							
	NADL-MD		C24V		NY-1		IND-46		SAN-78		MER-171		CG-1220	
	TI	IF	TI	IF	TI	IF	TI	IF	TI	IF	TI	IF	TI	IF
-1	4/4*	+	4/4	+										
-2	4/4	+	4/4	+	0/4**		0/4		0/4				0/4	
-3	4/4	+	2/4	+	0/4		0/4		0/4		0/4		0/4	
-4	3/4	+	0/4	-	0/4	+	0/4	+	0/4	+	0/4	+	0/4	+
-5	2/4	+	0/4	-	0/4	+	0/4	+	1/4	+	0/4	+	0/4	+
-6	0/4	-	0/4	-	2/4	-	1/4	-	3/4	-	0/4	+	3/4	-
-7	0/4	-	0/4	-	3/4	-	4/4	-	4/4	-	3/4	-	4/4	-
TCID ₅₀ /ml	10 ^{6.47}		10 ^{3.70}		10 ^{6.93}		10 ^{7.03}		10 ^{6.25}		10 ^{7.40}		10 ^{6.40}	

*Ratio of infected BEK cell cultures showing CPE over total cell cultures inoculated.

**A zero indicates no CPE by the challenge virus because the cells have been previously infected by the NCP virus and, therefore, show the interference phenomenon upon challenge with a CP virus.

fluorescein-tagged antiserum, only one coverslip per dilution of virus was employed to titrate the viruses, but several coverslip cultures per dilution could be stained with labeled globulin in order to calculate a TCID₅₀ by the method of Reed and Muench (15), in the same manner as was determined for the infected and non-infected BEK tissue culture cells (TI columns of Table 3). This immunofluorescence titration could provide an alternate method for titrating NCP viruses, in cases where it may not be desirable, nor feasible, to employ the challenge virus necessary in the interference titration method. A CP virus may not always be available for an interference titration, or certain laboratories may not want to risk introducing a contaminating virus into the viral system being titrated.

An inference can be made from the data presented in Table 2 that the NCP viruses form an antigenically related group of BVD viruses. Evidence for such a relationship is that the SN titer of the anti-NCP viral serum against each NCP virus (Column D of Table 2) is higher than the SN titer of the anti-CP viral serum against the same virus (Column B of Table 2). Furthermore, the intensity of fluorescence of NCP viral cultures stained with fluorescein-conjugated anti-NCP viral serum globulins (Column C of Table 2) is greater than the intensity of fluorescence of the same viral cultures stained with fluorescein-conjugated anti-CP viral serum globulins (Column A of Table 2). Conversely, the staining intensity of CP viruses is less with fluorescein-labeled anti-NCP

viral serum globulins (Column C) than it is with anti-CP viral serum globulins (Column A). This staining intensity phenomenon could be due to the availability of antigen receptor sites on infected cells which provide more coupling sites for labeled antibody from homologous virus systems. The SN titers of the NADL-MD virus conforms to this theorized antigenic relationship, but those of the C24V do not. The C24V virus is very difficult to titrate accurately because only partial CPE is exhibited in some BEK cells when SN tests are performed. This incomplete CPE could also explain the extremely low SN titers of 120 and 218 in Table 2 as compared with titers of 3365 and 1289 for the NADL-MD virus.

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A Simple Chemical Method for the Detection of Leaks in Flexible Isolators

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Flexible film isolators suitable for rearing and maintaining germfree animals first described by Trexler and Reynolds (1) have the advantages of lower costs and adaptability over those previously described (2). The flexible plastic films are inexpensive, easy to see through, and their flexibility permits limited movement of the contents of the isolator through any portion of the wall without disturbing the contamination barrier. The isolators are easily sterilized (3).

This report describes an alternative, inexpensive, easy method of detecting leaks in a flexible isolator. Trexler and Reynolds (1) reported that leaks could be detected by swabbing the suspect area with soap or detergent solution while the flexible isolator is under pressure. However, the air pressure must overcome the capillary force of the solution, to form a bubble, and the air pressure as maintained in plastic isolators is not great enough to detect small leaks. The Freon Leak Detector type H-1 made by the General Electric Co. of Schenectady, N.Y. or tests for electrical leakage are sensitive methods but expensive equipment is necessary (1). Even so, Trexler (4) states that small leaks in flexible film isolators are best detected

by the convenient and sensitive halogen leak detector. Flexible film isolators have been in wide use for a number of years but detection of leaks still is one of the difficult problems and the system must be leak proof to operate at maximum efficiency.

Method and Materials:

Close all the openings in the isolator except the air inlet. Introduce 1.5 ml. of concentrated ammonium hydroxide into the isolator and inflate it with air under pressure (1.5 in. of water). Allow ten minutes for the ammonium hydroxide vapours to saturate the atmosphere of the isolator, then take a glass rod dipped in concentrated hydrochloric acid and move it around all the seams and joints. Where a leak occurs dense white fumes of ammonium chloride are formed. On completion of the test the isolator is flushed with air to remove ammonium hydroxide vapours.

The test should be conducted in a well ventilated room.

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