Detection of Infectious Bovine Rhinotracheitis and Bovine Viral Diarrhea Viruses in the Nasal Epithelial Cells by the Direct Immunofluorescence Technique

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ABSTRACT

Nasal epithelial cells were collected by cotton swabs for the diagnosis in experimental and field cases of infectious bovine rhinotracheitis and field cases of bovine viral diarrhea in calves. A portion of the cells was washed twice in phosphate buffered saline and a 25 μL drop was placed on microscope slides. The cells were dried, fixed and stained according to the direct fluorescent antibody technique. Another portion of the same specimen was inoculated onto primary bovine skin cell cultures for virus isolation. In the experimental studies for infectious bovine rhinotracheitis, 29/35 specimens were positive by fluorescent antibody technique and 32/35 by cell culture and in the field cases, 22/119 were positive by fluorescent antibody technique and 19/119 by cell culture. In the field cases of bovine viral diarrhea, 28/69 samples were positive by fluorescent antibody technique and 14/69 by cell culture. When fluorescent antibody technique was performed on inoculated cell cultures a total of 24/69 specimens were positive for bovine viral diarrhea. The sensitivity of fluorescent antibody technique was thus comparable to that of cell culture method for infectious bovine rhinotracheitis and bovine viral diarrhea.

RÉSUMÉ

Cette expérience consistait à récolter des cellules de l'épithélium nasal, chez des veaux, à l'aide d'écouvillons de coton, dans le but de les utiliser pour diagnostiquer des cas expérimentaux et spontanés de rhinotrachéite infectieuse bovine, ainsi que des cas spontanés de diarrhée à virus bovine. Une suspension de ces cellules subit deux lavages, dans une solution physiologique-tampon phosphate (pH 7.2), avant qu'on l'applique sur des lames, à raison de 25 μL par lame. On laissa sécher ces préparations pour ensuite les fixer et les teindre selon la méthode de l'immunofluorescence directe. Le surnageant d'une partie de la suspension précitée servit par ailleurs à inoculer des cultures primaires de cellules cutanées bovines, pour l'isolation de virus.

L'immunofluorescence directe identifia 29 des 35 cas expérimentaux de rhino-trachéite infectieuse bovine, tandis que la culture cellulaire en identifia 32. L'immunofluorescence directe donna des résultats positifs avec 22 des 119 échantillons suspects, soumis par des praticiens, contre seulement 19, pour la culture cellulaire. En ce qui concerne les cas spontanés de diarrhée à virus bovine, l'immunofluorescence directe donna des résultats positifs avec 28 des 69 échantillons suspects, contre seulement 14, pour la culture cellulaire.

La sensibilité de l'immunofluorescence directe se révéla par conséquent comparable à celle de la culture cellulaire, pour le diagnostic de la rhinotrachéite infectieuse et de la diarrhée à virus bovines.

INTRODUCTION

Because of high incidence of respiratory infections in domestic animals, veterinary diagnosticians need a rapid method for the laboratory diagnosis of acute respiratory infections. The fluorescent antibody technique (FAT) offers the possibility of demonstrating viral antigens in the cells of the respiratory tract and has been applied in human virology for the diagnosis of influenza infection (1, 5,6,7,11), respiratory syncytial virus (RSV) (3,10) and parainfluenza (PI) virus (4,8). Good correlation between the results of FAT and cell culture technique (CCT) for virus isolation or serology has also been reported (1,4,5,6,7,9), but the occurrence of positive FAT with negative CCT or serology has also been confirmed (12,13). Using smears of nasal swabs, FAT has been applied for the diagnosis of infectious bovine rhinotracheitis (IBR) in cattle (13) and feline viral rhinotracheitis (2).

This paper presents the results

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of an experimental study to investigate the use and reliability of FAT on nasal epithelial cells for the diagnosis of IBR and its application for the field diagnosis of IBR and bovine viral diarrhea (BVD) in cattle.

MATERIALS AND METHODS

EXPERIMENTAL INFECTION WITH IBR VIRUS

Experimental Animals — Six calves free from antibody to IBR virus were purchased at eight to 12 weeks of age, from a commercial source. The animals were housed in pairs in isolators and observed for ten days. During this time, nasal secretions were taken for virus detection by the CCT and FAT and preinoculation blood was taken for detection of antibody in serum.

Inoculation of Animals — Four animals were each inoculated intranasally with $5 \times 10^{6.5}$ tissue culture infective doses of IBR virus (Cooper strain). Two nonexposed calves were kept as controls and were housed separately. The animals were observed for 20 days, during which time nasal secretions were collected from infected and control animals at postinoculation days (PID) 2-5, 7, 9, 11, 15 and 20. Blood was taken at PID 20 for detection of seroconversion to IBR virus.

Collection of Nasal Secretions — Nasal secretions were collected by brushing cotton swabs against the walls of the nasal passages. Two swabs used for virus isolation, were placed into 2 mL of Eagle's minimum essential medium (EMEM) containing 200 μg/mL kanamycin and streptomycin and 200 U/mL penicillin (Gibco Laboratories, Grand Island, New York) and stored at -20°C pending use. One swab used for FAT was placed in 2 mL of EMEM and processed within six hours.

Fluorescent Antibody Technique — The cells were removed from the swabs and dispersed in the medium by shaking in a Vortex shaker. After centrifugation of the suspension at 200 × g for eight minutes, the supernatant was decanted and the cells were washed in 5 mL of phosphate buffered saline (PBS) pH 7.2 and centrifuged twice. The cells were resuspended in PBS to a concentration of approximately 400 000 cells/mL. Clumps of cells were removed by a pipette prior to placing 25 μL of the suspension into each well of an eight well multitest slide (Flow Laboratories, Montreal, Quebec). The slides were air dried at room temperature and fixed in acetone for five minutes. They were rinsed briefly (five seconds) in deionized water to remove excess salts and dried again. The prepared specimens were stained immediately or stored at -20°C until used.

Fluorescein-conjugated rabbit antiserum to IBR (Microbiological Associates, Bethesda, Maryland) was diluted 1:16 in PBS and a drop of 20 μL of the diluted conjugate was placed into each of four wells. Two other wells were treated similarly with either conjugated rabbit antiserum to BVD (Microbiological Associates, Bethesda, Maryland) or conjugated antiserum to respiratory syncytial virus (RSV) as controls. One well was pretreated with hyperimmune rabbit anti-IBR serum in a fluorescence blocking test and one was left as untreated control. The slides were incubated for one hour at 37°C in a humid chamber.

After incubation, the slides were washed in three changes of PBS for 20 min each change and dried at room temperature. Cover-slips were mounted onto the slides in 85% glycerine-PBS pH 8.5 and the slides were examined with an incident light microscope (SM-LUX, Wild Leitz, Ottawa, Ontario). A sample was considered positive when at least two virus-specific fluorescent cells were observed in any one of the test wells.

Virus Isolation on Cell Cultures — Primary fetal bovine skin cell (FBSC) cultures at the second to fourth passage level, were grown in Linbro microtissue culture plates (Flow Laboratories, Montreal, Quebec) in EMEM supplemented with 5% fetal bovine serum (FBS), 200 U/mL penicillin and 200 μg/mL streptomycin. Confluent monolayers were inoculated with nasal secretions from the calves as follows: The frozen swabs in EMEM were thawed at 37°C in a water bath and shaken in a Vortex shaker. The suspensions were clarified by centrifugation at 400 × g for ten minutes and the supernatant inoculated onto the cell cultures using 0.1 mL/well, four wells/inoculum. After incubation for one hour at 37°C the inoculum was decanted and 0.2 mL EMEM containing 2% FBS was added to each well. Control cultures were inoculated with cell culture medium instead of nasal secretions.

The virus reisolated from each calf inoculated with IBR was identified by the serum neutralization test using hyperimmune rabbit anti-IBR serum. Rabbit anti-BVD, -PI3 and -RSV were used as control sera.

DIAGNOSTIC APPLICATION

During the past two and one-half years, 119 nasal swabs taken from cattle and submitted to this laboratory for diagnosis were examined by the direct FAT and by virus isolation in cell cultures. In the field, the nasal swabs were placed in 2-3 mL EMEM with antibiotics as for the experimental calves and delivered to the laboratory on the same day. All of the 119 specimens were tested for IBR virus and only 69/119 were tested for BVD virus.

On arrival, the specimens were shaken in a Vortex shaker to disperse the cells and the suspension was divided into two portions. One portion was centrifuged, the cells were washed and centrifuged twice, then subjected to the FAT using anti-IBR or -BVD conjugates. The other portion was inoculated onto FBSC cultures, grown in 24-well Linbro tissue culture plates, for virus isolation. The virus isolated was identified by the serum neutralization test. Negative and positive cell cultures were passaged once more in FBSC cultures and the inoculated cultures were studied by direct FAT in

order to determine the presence of BVD virus.

Paired serum samples were obtained from the same animal at approximately two and 21 days after the appearance of the clinical signs. Antibody titers to IBR and BVD viruses were determined by the serum-virus neutralization test. Seroconversion was considered as a fourfold increase in antibody titer.

RESULTS

MORPHOLOGY OF FLUORESCING CELLS

The appearance of the fluorescent nasal epithelial cells from calves infected with IBR virus is shown in Fig. 1. The fluorescence was nuclear-membrane-associated (Figs. 1B, C, D) or intranuclear and cytoplasmic (Figs. 1E, F). In some cases, granular fluorescence covering the entire cell was observed (Fig. 1F). Nuclear membrane-associated fluorescence was most frequent on PID 2 but in general granular intranuclear and cytoplasmic fluorescence were predominant on all days.

At PID 2-4, the nasal cells collected were mostly mature epithelial cells (Fig. 2A). At PID 5-20 basal or intermediate cells (Fig. 2B) became predominant. Compared to the mature epithelial cells, the latter cells showed diffused nonspecific fluorescence which was clearly different from

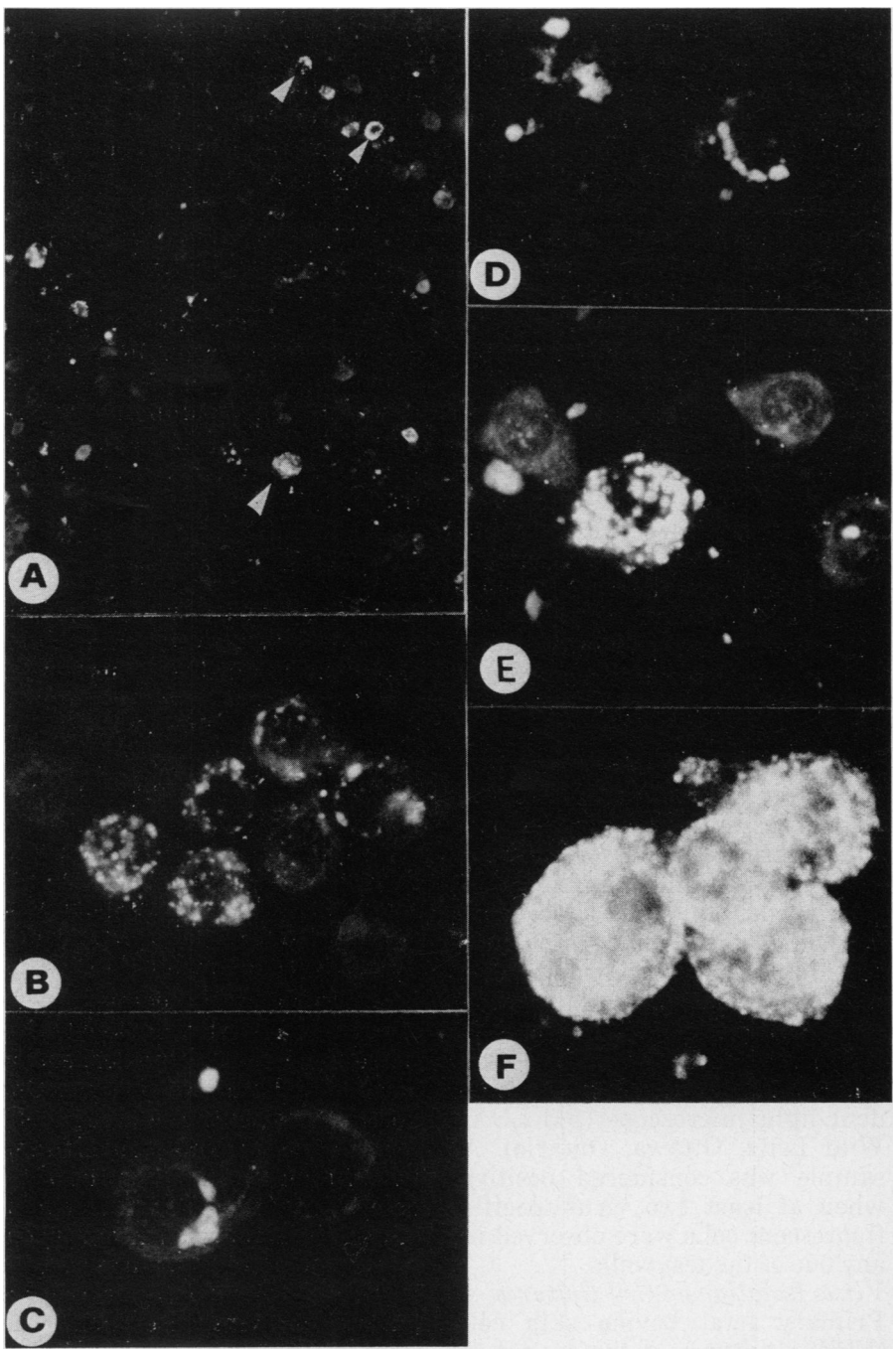

Fig. 1. Nasal epithelial cells at various stages of infection by IBR virus after staining by FAT. (A) — Overall view (arrows indicate some positive cells). X115. (B) — Granular fluorescence in a group of cells at PID 2. X520. (C and D) — Nuclear membrane associated fluorescence at PID 2 and 3 respectively. X740. (E) — Granular fluorescence in the nucleus and cytoplasm of an infected cell at PID 4. X520. (F) — Fluorescence in a group of cells at PID 3. X1100.

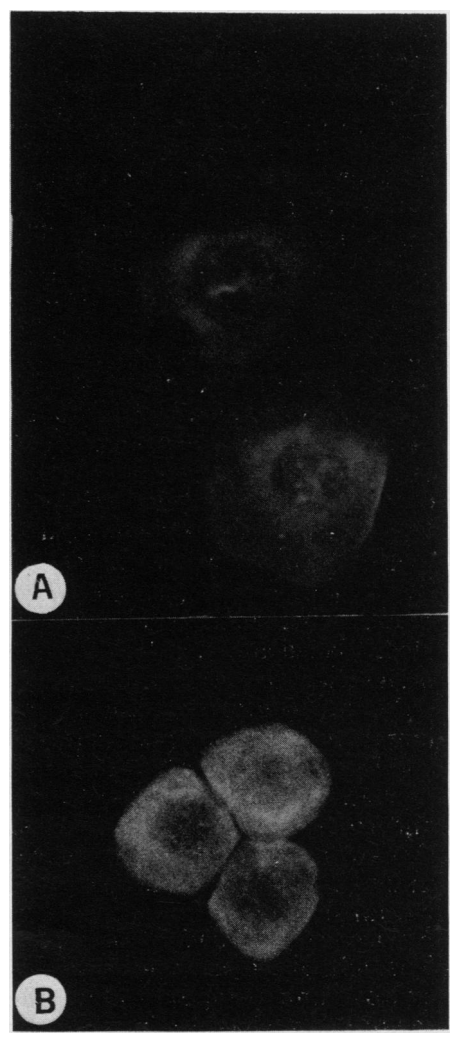

Fig. 2. Noninfected (A) mature and (B) immature nasal epithelial cells. X660.

the bright, granular and localized virus-specific fluorescence.

The fluorescence of nasal epithelial cells infected with BVD virus is shown in Fig. 3. The fluorescence was localized only in the cytoplasm. A similar type of fluorescence was observed in cell cultures infected with BVD virus.

VIRUS ISOLATION AND FAT

No virus was demonstrated in the nasal secretions of any of the experimental calf nor was antibody to IBR detected in the sera of these animals. The results comparing IBR virus isolation in cell culture to the demonstration of viral antigen by FAT after infection are given in Table I. Except at PID 7, the FAT detected virus with equal sensitivity to the cell culture technique. On the whole, the FAT was positive 29 times (90.6% sensitivity) compared to 32 (100%) for the CCT. No false positive immunofluorescence was observed, and all of the IBR virus inoculated calves seroconverted by PID 20. No fluorescence was observed in nasal cells from control calves, nor was virus isolated by cell culture. Control calves did not seroconvert.

DIAGNOSTIC FIELD CASES

The test results on field specimens diagnosis of IBR and BVD by FAT, CCT and FAT on cell cultures are given in Table II. For IBR, 22 out of 119 specimens tested were positive by FAT and of the 22, 19 were positive by CCT. The animals from which the 22 specimens were taken developed antibody to IBR, thus confirming IBR infection.

For BVD, 28 out of 69 specimens tested were positive by FAT. Of the 28, 14 were positive by CCT and 14 were negative. When FAT was performed on cell cultures, 24 of the 28 FAT positive specimens were identified as positive. Seroconversion was detected in all the positive animals, except three which were not tested (Table II). Coinfection by IBR and BVD viruses was detected by FAT in three animals (Table II).

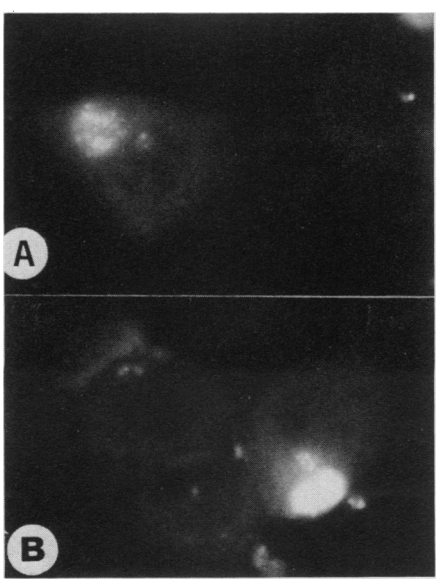

Fig. 3. Nasal epithelial cells infected with BVD virus showing cytoplasmic fluorescence. X770.

TABLE I. Fluorescent Antibody Technique (FAT) Compared to Cell Culture Technique (CCT) in Detecting IBR Virus in Nasal Secretions of Experimentally Infected Calves

	Postinfection day									
	2	3	4	5	7	9	11	15	20	TOTAL
FAT	4/4	4/4	4/4	4/4	1/4	3/3	4/4	4/4	1/4	29/35
CCT	4/4	4/4	4/4	4/4	4/4	3/3	4/4	4/4	1/4	32/35

DISCUSSION

Immunofluorescence technique is commonly used in many laboratories to demonstrate viral antigens in tissue sections or inoculated cell cultures. Fluorescent antibody technique on infected cell cultures sometimes requires two passages of the virus in cell cultures. This is a costly and time consuming procedure. Our results confirmed the findings of previous investigators (2, 4, 5, 6, 7, 14) that FAT can be applied directly on smears of swabs from nasal passages of infected animals. In our technique, we used washed suspension of nasal epithelial cells instead of smears. This has the advantage of eliminating cell debris as well as mucous, thus reducing the background fluorescence and intensifying the virus specific fluorescence.

In the diagnosis of field cases of IBR and BVD, FAT was slightly more sensitive than cell culture method. Because most of the specimens were delivered directly to our laboratory within the same day, the epithelial cells were intact at the time of preparation of the cells for FAT. This probably enhanced the specificity and sensitivity of the FAT.

The use of FAT in the diagnosis of BVD has a special significance because of the presence of noncytopathic strains of the virus in the field. For BVD virus detection, results indicate that FAT is twice as sensitive as cell culture method when cytopathic effect is considered as an indicator. But when FAT is performed on inoculated cell cultures, equality is almost reached between the sensitivities of FAT and CCT. Considering the overall cost involved in CCT and FAT on cell culture it is preferable

TABLE II. Fluorescent Antibody Technique (FAT) Compared to Cell Culture Technique (CCT) in the Diagnosis of Field Cases of IBR and BVD

Year	No. of animals tested	No. positive			
		FAT	CCT	FAT on CC	Seroconversion
IBR					
1979	50	9	8	NT	10
1980	60	10	8	NT	11
1981	9	3[a]	3	NT	NT
TOTAL	119	22	19	NT	22
BVD					
1979	60	24	14	24	25
1981	9	4[a]	0	0	NT
TOTAL	69	28	14	24	25

[a]Three animals were positive for IBR and BVD
CC = Cell culture
NT = Not tested

to use direct FAT on nasal cells. However, if the specimens arrive in a state of deterioration in which most cells of the nasal swabs are disintegrated, then preference should be given to the CCT. Immunofluorescence also detected coinfection of IBR and BVD viruses which could not be detected by cell culture method possibly because of interference or the slow growth of BVD virus.

In conclusion, the sensitivity and reliability of direct immunofluorescence technique for the diagnosis of IBR and BVD using nasal epithelial cells are comparable to and in some cases better than the cell culture isolation method. The technique is rapid thereby reducing the diagnosis time from two to five days to a few hours.

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