A STUDY OF BOVINE RESPIRATORY SYNCYTIAL VIRUS USING INDIRECT IMMUNOFLUORESCENCE AND COMPLEMENT FIXATION

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1. Specific Cytoplasmic Fluorescence of BRSV Antigen in GTU Cells. Mag. X312.5. 28

CHAPTER I

INTRODUCTION

Bovine respiratory syncytial virus (BRSV) has been isolated in several parts of the world and has been found to be a major cause of bovine respiratory disease (1,2,3,4,5,6). The serological surveys that were done with these studies indicate that BRSV is widespread in many bovine populations.

Serological surveys have been done using serum neutralization, plaque reduction, complement fixation or fluorescent antibody tests. Serum neutralization and plaque reduction are useful techniques, but they require several days to get results (1,2,3,4,5,6,7). The complement fixation (CF) test is quick and easy to perform, but details of the methods have not been described (1,8). A more recently developed test, that is sensitive and easy to perform, is the indirect fluorescent antibody (IFA) test (9).

The serological studies done so far have been limited to random single samples of paired serum samples. This kind of testing has indicated the incidence of BRSV and its role in respiratory disease outbreaks, but has not given us much information about the antibody response in cattle several months after infection.

In the present study, the antibody response of naturally infected calves is followed through a four month period, using the IFA and CF test. Other objectives of this study were to determine the sensitivity

of the IFA technique in performing BRSV virus titrations, and to evaluate a microtiter plate, IFA method for detecting serum antibody titers to BRSV.

CHAPTER II

LITERATURE REVIEW

Respiratory syncytial virus (RS virus) is a virus of considerable importance in human medicine. Since its isolation in 1957, a considerable amount of research has been done with this virus and it is now known to be a major cause of human respiratory disease, particularly among infants and young children (10). It was through research with human RS virus that the closely related bovine RS virus was discovered.

The first evidence that a bovine RS virus might exist came in 1963. A group of researchers working with the RS virus realized that the calf serum used in their tissue culture medium was inhibiting syncytium production that is characteristic of RSV. Further studies of different lots of calf serum established this inhibition to be caused by a specific antibody. This led them to the conclusion that a virus similar to the human RS virus must be present in cattle (11).

Paccaud and Jaquier (1) reported the first isolation of BRSV from Swiss cattle in 1970. They reported on a respiratory disease outbreak that lasted 3 to 10 days. The cattle had upper respiratory tract symptoms, but several head later developed bronchopneumonia. They isolated a virus that was serologically related to RSV and they found seroconversions to the virus they isolated in several head of cattle using the complement fixation and serum neutralization tests. Their virus was also similar to RSV in its physicochemical properties. It was inacti-

vated at low pH and at 56 C, but stable at -70 C and it was sensitive to lipid solvents and its size was between 0.22 u and 0.45 u.

Bovine RS virus was next isolated in Japan during an outbreak of acute bovine respiratory disease. Inaba and coworkers (2) conducted a serological study and found BRSV to be the cause of this outbreak, and they also found their bovine RS virus to be serologically similar to RSV. In a later study (12), they found it contained RNA, was labile at 56 C and 37 C, but stable at -80 C, and it was inactivated by trypsin and deoxycholate.

Bovine RS virus was also isolated in 1970 during an outbreak in Belgium (3), and in England in 1971 (4). Forty percent of the Belgium cattle tested had neutralizing antibodies to BRSV, and they found that BRSV was the most common agent in respiratory disease in cattle during the cold months of the year (13,14).

Berthiaume (8) tested sera from several species of animals in Canada by complement fixation. However, he did not give any details of his test methods. He suggested that sheep may make a good experimental model for studying BRSV.

Smith (5) was the first to isolate BRSV in the United States. He isolated it from a herd of cattle in Iowa during an acute outbreak of respiratory disease. The most common symptoms were high rectal temperatures and nasal discharges. His findings agreed with earlier reports that BRSV is closely related to, but not identical to RSV, both serologically and in its physicochemical properties. His serological survey indicated that exposure to BRSV was very common among Iowa cattle.

Rosenquist (6) recovered a bovine RS virus at about the same time in Missouri from two calves with acute respiratory disease. He found

high convalescent, antibody titers to the viruses he isolated and he was also able to demonstrate cross neutralization with antiserum to the Long strain of RSV.

Researchers in Alabama conducted a serological study to determine the prevalence of BRSV in that state (7). They found a positive neutralizing titer to the Long strain of RSV in 60 percent of the adult healthy cattle they tested. They also found many seroconversions in three herds when these calves were exposed to other calves with respiratory disease.

Most of the work done with BRSV has been done using the neutralization test. However, Takahashi and coworkers (15) used the complement fixation test for detecting serum antibodies to BRSV. This required a modification of adding five percent normal calf serum to the complement diluent. They found a linear correlation between the complement fixation and neutralization tests.

Potgieter (9) developed an indirect fluorescent antibody test for the diagnosis of BRSV. He was able to detect fluorescence at 48 hours and some fluorescence at 72 hours, but not at 96 hours after infection. Also he found antibody titers in some 67 percent of 400 bovine sera in Oklahoma cattle and commented that this technique was a rapid, sensitive and a relatively simple procedure to perform.

In summary, BRSV has been found to be a major cause of bovine respiratory disease in several countries. It is similar to, but not identical to, RSV and it contains RNA, is sensitive to lipid solvents and low pH, and its size is between 0.22 u and 0.45 u. Clinical symptoms vary from mild upper respiratory symptoms to severe bronchopneumonia. Cross reactivity has been demonstrated between RSV and BRSV by complement fixation and serum neutralization. This cross reactivity has

been used to demonstrate antibodies to BRSV by some researchers. A indirect fluorescent antibody method has also been used for detecting BRSV antibodies. Since it is a quick and simple procedure, it offers some advantages over the CF and neutralization tests.

CHAPTER III

MATERIALS AND METHODS

Cell Cultures and Virus

Cell Culture Technique

The cell culture line used in all aspects of this project was a secondary culture of goat turbinate cell (GTU). The initial culture was obtained at passage level ten from the Oklahoma Animal Disease Diagnostic Laboratory, Stillwater, Oklahoma, and turbinate cells from another goat were used in later aspects of the research beginning at passage level seven.

These cells had been preserved in 7.5 percent dimethyl sulfoxide (DMSO) and stored in one ml aliquots at -70 C. The cells were used by rapidly thawing the vials at 37 C, then suspending the cells in ten ml of growth medium. The viable cells were allowed to attach overnight, then the medium was replaced with fresh growth medium. All the cells were grown in 75 cm^2 or 25 cm^2 plastic disposable tissue culture flasks (Corning Glass Works, Corning, N.Y.), and incubated at 37 C in a humidified CO_2 incubator with five percent CO_2.

The growth medium used was Eagles basal medium with Earles salts and L-glutamine (BME/E). It was buffered with three ml of a 7.5 percent $NaHCO_3$ solution and supplemented with ten percent, irradiated, heat inactivated, fetal bovine serum (Flow Laboratories, Rockville, Md.). The

cell cultures were maintained with BME/E medium supplemented with two percent fetal bovine serum. Cultures were generally kept antibiotic free, but when necessary, 50 to 100 μg of gentamicin (Schering Corp., Kenilworth, N. J.) was added to the medium. The BME/E medium for cell cultures grown in microtiter plates and chamber/slides was supplemented with four percent fetal bovine serum and 100 μg of gentamicin per ml of medium.

When it was necessary to pass cells, the medium was removed from the flasks and the monolayers were rinsed three or four times with Rinaldini enzyme solution (R-saline) (16). The monolayers were then trypsinized with a 0.05 percent trypsin (Miles Laboratories, Inc., Elkhart, Ind.) solution. After the cells had loosened from the flask, the trypsin was neutralized with growth medium, then the cells were split 1:4 by passing cells into three new flasks and retaining the original flask. To further remove the trypsin and non-viable cells, the medium was replaced with fresh growth medium the next day.

Stock Virus

The virus used was the FS1-1 strain of bovine respiratory syncytial virus (BRSV), obtained from, and originally isolated at Iowa State University (5). The virus had been passed several times in cell cultures containing antibodies to bovine diarrhea virus (BVD) and it was found to be free of BVD.

Growth of Virus in Cell Cultures

The GTU cells were infected either by passing the tissue culture medium from an infected flask to uninfected GTU cells, or the infected

cells were scraped loose, then the cells and medium were passed to uninfected GTU cells.

The virus was harvested with the cells when the cells were showing 75 percent or greater cytopathic effect (CPE). The cells were scraped from the surface with a sterile rubber policeman, then the virus was either passed to uninfected flasks or placed in sterile test tubes for storage.

Virus intended for storage was preserved by adding autoclaved DMSO (Fisher Scientific Co., Fairlawn, N.J.) to the virus suspension. After the cells were scraped loose, enough DMSO was added to equal a final concentration of ten percent, and the virus divided into two ml aliquots and stored at -70 C.

Virus Titrations

Virus titrations were done using four and eight well chamber/slides (Lab-Tek Products, Miles Laboratories, Rockville, Md.). GTU cells were trypsinized and the cells were suspended in growth medium, then the cells were centrifuged and this medium was removed. The packed cells were diluted with BME/E to contain approximately 2.7×10^5 cells per ml. Eight chambered slides were seeded with 0.2 or 0.15 ml of this cell suspension and four chambered slides were seeded with 0.3 ml of cell suspension.

The virus was removed from storage at -70 C, and ten-fold dilutions of the virus was made in BME/E medium. Four wells of GTU cells were infected, at the time of seeding, with either 0.1, 0.2 or 0.25 ml amounts of each virus dilution. With each titration, four chambers were left uninfected to serve as controls. The slides were incubated at 37 C in

a humidified CO_2 incubator (five percent CO_2) for 30, 72, 120 (five days), or 168 hours (seven days). For those slides incubated for five or seven days, the medium was changed on the third day of incubation.

After incubation, the slides were fixed and stained either by a May-Grunwald-Giemsa (MG-G) method or an indirect fluorescent antibody (IFA) method. In one trial, the slides were first stained and examined by the IFA method then stained and examined by the MG-G method.

The Reed-Muench (17) method, without summation, was used to calculate the 50 percent tissue culture infective dose ($TCID_{50}$).

The May-Grunwald-Giemsa Staining Method

The medium was removed and the cells were rinsed three times in R-saline, then the cells were fixed for five minutes in absolute methanol. The plastic chambers were removed and the slides were covered with May-Grunwald stain for ten minutes, followed by 20 minutes of staining with Giemsa stain. Next, the slides were rapidly dehydrated with two quick changes of acetone, then cleared with three changes of acetone-xylene, 2:1; and three changes of acetone-xylene, 1:2. Finally the slides were covered with fresh xylene for ten minutes, then cover slips were mounted using permount.

The slides were examined using the low and high dry objective of a light microscope. A chamber was considered infected if one or more syncytia (giant cells), containing three or more nuclei and cytoplasmic inclusions, were observed.

Immunofluorescent Staining Method

For those slides intended for immunofluorescent staining, the

plastic chambers were removed and the slides were rinsed in 0.01 M phosphate buffered saline (PBS), pH 7.5, then fixed in acetone for ten minutes at room temperature. After fixation the slides were stored at -70 C until they were stained.

A positive serum (from calf 115 of the C-8 project, March 8, 1976) and a negative serum (irradiated fetal bovine serum; Miles Laboratories, Rockville, Md.) were used for the initial step of the staining procedure. The positive serum was used to cover all the infected cells and two uninfected wells, and the negative serum was used to cover the remaining two control wells.

The cells were covered with a few drops of serum then incubated at 37 C in a humidified chamber for 30 minutes. This was followed by rinsing 20 minutes in three changes of PBS and one change of distilled water, then the slides were allowed to partially dry at room temperature. Next, the cells were covered with rabbit, antibovine IgG, FITC conjugate (Miles Laboratories, Inc., Research Division, Elkhart, Ind.), diluted 1:8 in PBS. The cells were again incubated for 30 minutes at 37 C in a humidified chamber, then rinsed and dried as before. Cover slips were then mounted using mounting fluid containing nine parts PBS and one part glycerol.

The slides were then examined with a fluorescent microscope for the presence of specific, cytoplasmic fluorescence. A well was considered positive if at least one fluorescing cell was observed.

Immunofluorescent Techniques

Preparation of Chamber/Slides

A modification of Potgieter's (18) method was used to prepare

chamber/slides. GTU cells from a 75 cm^2 tissue culture flask were trypsinized and suspended in 15 ml of growth medium. Five ml of this suspension was used to make four, eight or four chambered, chamber/slides. The cell suspension was placed in a sterile screwcap test tube, and centrifuged at 2000 rpm for five minutes.

After centrifuging, the growth medium was removed and the cells resuspended in eight ml of BME/E (containing four percent fetal bovine serum and gentamicin), giving a cell suspension of approximately 2.5 x 10^5 cells per ml. Two ml aliquots of virus, containing approximately 10^4 $TCID_{50}$/ml, were removed from storage and thawed, then mixed with the cell suspension. Eight chambered slides were inoculated with 0.3 ml of this suspension per chamber and four chambered slides were seeded with 0.6 ml of the suspension per chamber. The slides were then incubated in a humidified CO_2 incubator at 37 C.

At the end of 30 hours incubation, the slides were rinsed in 0.01 M PBS and fixed in acetone for ten minutes. The slides were then stored at -70 C until needed.

Preparation of Microtiter Plates

Disposable, 96 well tissue culture, microtiter plates (Linbro Scientific Co. Inc., Hamden, Conn.) were also used in immunofluorescent studies. GTU cells were trypsinized and infected the same as the chamber/slides, then 0.1 ml of the cell suspension was added to each well of the plates. An additional 0.05 ml of medium was added to each well, then the plates were incubated in a CO_2 incubator at 37 C for 30 hours.

After incubation, the plates were fixed by the method outlined by

Pursell and Cole (19). The medium was aspirated off with a Pasteur pipet-suction flask apparatus and each well was rinsed with PBS, then one or two drops of PBS were added to each well and the wells were carefully filled approximately three-fourths full with acetone. Each well was allowed to fix in acetone at least ten minutes before the PBS-acetone mixture was removed. The plates were then stored at -70 C until they were stained.

Antibody Titration and Fluorescent Staining

Procedure

Potgieter's (18) indirect immunofluorescent staining procedure was used for staining the chamber/slides and microtiter plates. The sera to be tested was initially screened at a 1:10 dilution in PBS, and positive sera were further tested at two-fold dilutions from 1:20 through 1:640.

The staining procedure was started by covering the cells with a few drops of serum dilution. The preparations were then incubated at 37 C in a humidified chamber for 30 minutes. This was followed by rinsing 20 minutes with three changes of PBS and one change of distilled water, then the preparations were allowed to partially dry at room temperature.

Next, the cells were covered with rabbit, anti-bovine IgG, FITC conjugate (Miles Laboratories, Inc., Research Division, Elkhart, Ind.), diluted 1:8 or 1:10 in PBS. The cells were again incubated for 30 minutes at 37 C, followed by rinsing as before. The rinsing stages were done in coplin jars when slides were stained, and a Pasteur pipet-suction flask apparatus was used to remove reagents with the microtiter

plates.

The tissue culture plates were allowed to dry by inverting them on a paper towel. For the chamber/slides, the rubber gaskets were removed and the slides were mounted with coverslips using a mounting fluid containing nine parts PBS and one part glycerol.

Each time the staining procedure was done, a negative control serum (irradiated, fetal bovine serum; Miles Laboratories, Rockville, Md.) and a positive control serum (calf 115 from the C-8 project, March 8, 1976), diluted 1:10 in PBS, were included with the test.

Microscopy and Photography

The specimens were examined with transmitted light using a Lietz orthoplan microscope equipped with a dry darkfield condenser, a 150 watt high pressure xenon lamp, a LP 500 exciter filter and a K 510 barrier filter.

This microscope was used with a X20, long working distance objective and a X25 and X40 objective with X10 occulars giving final magnifications of X200, X250 and X400, respectfully. The long working distance objective was used to examine the microtiter plates and the other objectives were used to examine the chamber/slides.

Another microscope was used for photography and to examine some of the chamber/slides. This was a Carl Zeiss microscope equipped with epi illumination, a halogen lamp, and a 490-500 nanometer exciter filter and a 528 nanometer barrier filter. Two objectives, a X25 and a X40, were used with X12.5 occulars, giving final magnifications of X312.5 and X500.

The cells were examined for specific cytoplasmic fluorescence and

were considered positive if one or more fluorescing cells or syncytia were observed. At higher magnifications, this was observed as fluorescing particles, while at lower magnifications fluorescence was observed as complete or partial cytoplasmic fluorescence.

Photographs were taken using a Carl Zeiss, 35 mm camera and ansochrome (General Aniline Film Corp.) 500 ASA daylight color film.

Complement Fixation Techniques

Preparation of Complement Fixation Antigens

Two methods of preparing complement fixation antigens were used, one a whole culture antigen and the other a cellular antigen.

The whole culture method used was that of Beem and Hamre (10). Monolayers of GTU cells were infected with the FS1-1 strain of bovine RS virus, then incubated at 37 C until the cells were showing 75 percent or greater CPE. The remaining attached cells were scraped loose and the medium and cells were stored at -70 C, followed by several cycles of thawing at 37 C and freezing at -70 C. The medium was then centrifuged and the supernatant fluid retained as antigen. Antigen from several flasks were pooled and stored at -70 C.

Modifications in this procedure were also used. The antigen was concentrated 1:10 using a Millipore Molecular Separator (Millipore Corp., Bedford, Mass.), and media from cells showing less than 75 percent CPE were tested for CF antigens.

The other method of preparing antigens was a modification of Schmidt and Lennettes' method (20). The antigen was produced the same as the whole culture antigen, but instead of immediately freezing the medium, it was centrifuged and the cellular portion was saved. Then

the cells were resuspended in R-saline to one tenth the original volume. The cells were frozen (-70 C) and thawed (37 C) several times, centrifuged, and the supernatant fluid was used as antigen. Antigen from several flasks were pooled and stored at -70 C until needed.

The antigens were titrated by testing two-fold dilutions of antigen (1:2 to 1:64) against two-fold dilutions of antiserum (1:8 to 1:128). One unit of antigen was defined as the highest dilution of antigen showing 75 percent or greater fixation with the highest dilution of serum. Four units, or four times the one unit dilution, were used in the CF test (21).

When anticomplementary activity was encountered with the antigens, they were treated with inactivated guinea pig serum (22). Guinea pig complement was inactivated at 56 C for 30 minutes, then it was added, at a 10 or 25 percent concentration to the antigen. The mixture was then incubated at 37 C for one hour, and the antigen titrations were repeated.

The Complement Fixation Test

The complement fixation (CF) test used was the microtiter method of Lennette's (21), except that the two percent sheep cell suspension was determined by centrifuging the cells rather than by using a spectrophotometer.

The sheep cells, hemolysin and complement were all obtained from commercial sources (Brown Laboratories, Topeka, Kan.; Microbiological Associates, Bethesda, Md.; and International Scientific Industries, Inc., Cary, Ill., respectfully). Also, commercially prepared CF reagents for the Long strain of respiratory syncytial virus (Microbiological Associates, Bethesda, Md.) were used. These included a positive

serum (RSV antiserum, titer 1:64), a positive antigen (RSV antigen, titer 1:64), and a non-infected tissue culture control antigen.

The RSV antiserum was used as a positive control serum and fetal bovine serum (Flow Laboratories, Rockville, Md.) was used as a negative control serum. After titration, the RSV antigen was diluted to contain four units and was used in the CF test rather than the previously described bovine virus antigens.

The CF test was conducted using disposable "U" bottom plates (Linbro Scientific Co., Hamden, Conn.), 0.025 ml droppers (Flow Laboratories, Rockville, Md.), and 0.025 ml diluting loops (Baltimore Biological Laboratories, Cockeysville, Md.). In determining the CF antibody titer, serum was diluted serially two-fold and 0.025 ml of four unit antigen was added to each dilution, Then 0.025 ml of complement (containing two units) was added to each dilution and the plates were shaken then incubated overnight at 4 C. The next morning 0.05 ml of sensitized sheep cells was added to each well, then the plates were sealed with vinyl tape and incubated in a 37 C waterbath for 30 minutes. After incubation the plates were centifuged for ten minutes and examined. The antibody titer was expressed as the highest serum dilution showing 25 percent or less hemolysis.

When bovine serum was being used in the antigen titrations or the CF test, a modifying factor was added to the complement diluent. Modifier was also used in the complement titrations to account for any anticomplementary effects of the modifier. This modifier was fresh normal bovine serum (obtained from the Oklahoma Animal Disease Diagnostic Laboratory, Stillwater, Okla.) and was added at a five percent concentration to the complement diluent.

Anticomplementary serum was treated by adding 0.4 ml of serum to 0.1 ml of guinea pig complement, and incubating at 37 C for 30 minutes, then the mixture was diluted in saline diluent. The serum was heat inactivated at 56 C for 30 minutes, then retested (23).

Serological Survey

The serum samples tested were from 25 calves raised at the Veterinary Research Station at Pawhuska, Oklahoma. The calves were used in the C-8 bacterin experiment conducted by another research group (the Respiratory Disease Laboratory, Oklahoma State University), and the serum samples they collected were used in the present research with their permission. The calves were recently weaned, four to seven month old calves weighing 400 to 500 pounds. The experiment was conducted from November, 1975 through March, 1976, with serum specimens taken at approximately weekly intervals, and stored at 4 C. This experiment was divided into two parts referred to as the Pawhuska and Stillwater phase. A protocol for this experiment is attached to the Appendix.

CHAPTER IV

RESULTS

The results of the virus titration experiments are given in Table I. The sensitivity of an IFA titration method was determined by comparing this method to a MG-G titration method.

In the first experiment, the IFA titration was incubated for 30 hours and the MG-G titration was incubated for seven days (168 hours). At these incubation periods the IFA method was less sensitive (gave a lower titer) than the MG-G method. This was also the case in the second experiment when the same incubation times were used, but the size of the inoculum was increased from 0.1 ml to 0.25 ml.

In the third experiment, 0.2 ml of virus inoculum was used, and IFA titrations incubated for 72 and 120 hours were compared to a MG-G titration incubated for 168 hours. Both the IFA titrations gave titers that were 0.5 log higher than the MG-G method. In this case the IFA method was not only a more sensitive method, it also required a shorter incubation period by 48 hours.

In the last experiment, an attempt was made to eliminate any random variation between the two methods by first examining the slides by the IFA method, then restaining and examining these slides by the MG-G method. Two titrations were done using a 72 and a 120 hour incubation period. At 72 hours, the IFA method gave a slightly higher titer and this titer was the same titer obtained by both the IFA and MG-G method

TABLE I

SUMMARY OF VIRUS TITRATIONS

Date: Harvest[1]	Date: Titered	Method[2]	ml of Inoculum	Hours Incubation	Titer[3]
Feb. 16	Apr. 9	MG-G	0.10	168	$10^{4.67}$
Feb. 16	Apr. 9	IFA	0.10	30	$10^{4.5}$
Apr. 11	Apr. 25	MG-G	0.25	168	$10^{5.1}$
Apr. 11	Apr. 25	IFA	0.25	30	$10^{4.1}$
Apr. 17	May 12	MG-G	0.20	168	$10^{4.2}$
Apr. 17	May 12	IFA	0.20	72	$10^{4.7}$
Apr. 17	May 12	IFA	0.20	120	$10^{4.7}$
Apr. 24	May 23	MG-G	0.25	72	$10^{4.1}$
Apr. 24	May 23	IFA	0.25	72	$10^{4.3}$
Apr. 24	May 23	MG-G	0.25	120	$10^{4.3}$
Apr. 24	May 23	IFA	0.25	120	$10^{4.3}$

[1]Represents the date the virus was stored at -70 C.

[2]MG-G = May-Grunwald-Giemsa technique: IFA = Indirect Immunofluorescent Antibody Technique.

[3]Expressed as $TCID_{50}$/ml.

at 120 hours incubation. This again indicated that the IFA method was as sensitive as the MG-G method, but required two less days incubation than the MG-G method.

The results of these experiments indicate that the IFA method may be a more sensitive method of determining BRSV titers and it requires a shorter incubation time than the MG-G method. However, when IFA titrations are done at 30 incubation, it is not as sensitive as MG-G titrations done at seven days.

Results of the fluorescent antibody titers are given in Tables II, III, and IV. All of these results were obtained by using microtiter tissue culture plates stained by an indirect fluorescent antibody procedure.

Table II gives the range of antibody titers, and the degree of fluorescence, of 24 sera from the last part of the C-8 experiment. This information was used to determine a starting dilution and the range of antibody titers. Undiluted serum gave too much nonspecific fluorescence and the results at a 1:1000 dilution were either borderline or negative, so a 1:10 starting dilution was used and all positive sera were tested at two-fold dilutions from 1:20 through 1:640.

The titers, and the degrees of fluorescence, of the Pawhuska and Stillwater sera are given in Tables II and III. Only one calf, number 127, had an antibody titer at the beginning of the experiment, and another calf, number 105, had a borderline reaction at this time. All the calves began to show an antibody rise in one week and continued to show a rise until a titer of 1:640 was reached within the first month (December 11) by all the calves. The titers and the degree of fluorescence began to decrease slightly by five or eight weeks (December 18

TABLE II

RANGE OF FLUORESCENT ANTIBODY TITERS FROM C-8 PROJECT USING MICROTITER PLATES

Calf No.	Date	Undiluted	Serum Dilutions 10	100	1000
102	Mar. 8	4+[1]	4+	2+	+/-
105	Mar. 8	3+	2+	1+	-
113	Mar. 8	3+	2+	1+	-
115	Mar. 8	4+	2+	1+	-
117	Mar. 8	3+	2+	2+	-
119	Mar. 8	3+	2+	2+	-
120	Mar. 8	3+	2+	1+	-
122	Mar. 8	2+	1+	1+	-
123	Mar. 8	4+	3+	2+	-
124	Mar. 8	3+	2+	2+	+/-
125	Mar. 8	3+	2+	2+	+/-
126	Mar. 8	4+	3+	+/-	-
127	Mar. 8	2+	2+	1+	-
128	Mar. 8	3+	2+	1+	-
114	Feb. 10	3+	2+	1+	-
121	Feb. 10	3+	3+	2+	+/-
122	Feb. 10	3+	3+	2+	+/-
125	Feb. 10	3+	2+	2+	-
127	Feb. 10	4+	1+	-	-
115	Feb. 23	3+	2+	1+	-
117	Feb. 23	3+	3+	2+	-
123	Feb. 23	3+	3+	2+	-

[1]Degree of fluorescence; 4+, very strong; 3+, strong; 2+, moderate; 1+, weak; +/-, borderline; (-), no fluorescence.

TABLE III

PAWHUSKA PROJECT ANTIBODY TITERS BY INDIRECT FLUORESCENT ANTIBODY TEST

Calf No.	Nov. 13	Nov. 20	Nov. 26	Dec. 4	Dec. 11	Dec. 18	Jan. 15
Group I							
101 S[1]	-	10(1+)[2]	160(1+)	640(1+)	640(1+)	640(1+)	640(1+)
102 S	-	20(1+)	160(1+)	640(1+)	640(3+)	640(3+)	640(2+)
103 S	-	20(1+)	640(1+)	640(1+)	640(1+)	640(1+)	640(1+)
104 C	-	10(1+)	160(1+)	640(2+)	640(2+)	640(1+)	320(1+)
105 C	10(+/-)	20(2+)	320(1+)	640(1+)	320(1+)	320(1+)	160(1+)
Group II							
107 S	-	20(1+)	160(1+)	320(1+)	640(1+)	320(1+)	320(1+)
108 S	-	40(1+)	640(1+)	640(1+)	640(2+)	640(2+)	640(1+)
109 S	-	20(1+)	160(1+)	320(3+)	640(2+)	640(1+)	640(1+)
110 C	-	40(1+)	160(1+)	640(1+)	640(1+)	640(2+)	640(1+)
111 C	-	10(1+)	640(2+)	640(3+)	640(2+)	640(2+)	640(2+)
Group III							
113 S	-	20(1+)	320(2+)	320(2+)	640(3+)	640(2+)	640(1+)
114 S	-	20(1+)	160(1+)	640(1+)	640(1+)	640(1+)	320(1+)
115 S	-	10(1+)	320(1+)	640(2+)	640(2+)	640(1+)	640(1+)
116 C	-	10(1+)	80(1+)	80(1+)	640(1+)	640(1+)	320(1+)
117 C	-	40(1+)	320(1+)	320(2+)	640(2+)	640(1+)	640(1+)

TABLE III (Continued)

Calf No.	Nov. 13	Nov. 20	Nov. 26	Dec. 4	Dec. 11	Dec. 18	Jan. 15
Group IV							
119 S	-	20(1+)	640(1+)	640(2+)	640(2+)	640(2+)	640(1+)
120 S	-	10(1+)	320(1+)	640(2+)	640(2+)	640(2+)	640(2+)
121 S	-	20(1+)	320(1+)	640(1+)	640(2+)	640(2+)	640(1+)
122 S	-	10(1+)	20(1+)	160(1+)	640(1+)	640(1+)	320(1+)
123 S	-	10(1+)	320(1+)	640(2+)	640(2+)	640(2+)	640(1+)
124 S	-	80(1+)	320(1+)	640(2+)	640(2+)	640(1+)	640(1+)
125 S	-	10(+/-)	320(1+)	640(1+)	640(1+)	640(1+)	640(1+)
126 C	-	10(1+)	320(1+)	640(2+)	640(1+)	160(1+)	160(1+)
127 C	10(1+)	40(1+)	80(1+)	320(1+)	640(2+)	640(1+)	640(1+)
128 C	-	20(2+)	320(1+)	640(2+)	640(1+)	640(1+)	640(1+)

[1]S = Calves shipped in truck; C = Calves not shipped (controls).

[2]Reciprocal of antibody dilution. Parentheses indicates quantitative degree of fluorescence. 4+, very strong; 3+, strong; 2+, moderate; 1+, weak; (+/-) borderline; (-), no fluorescence at 1:10 dilution.

TABLE IV

STILLWATER PROJECT ANTIBODY TITERS BY INDIRECT FLUORESCENT ANTIBODY TEST

Calf No.	Jan. 29	Feb. 10	Feb. 23	Mar. 8	Mar. 16
Group I					
104	320[1]	320	320		N.T.[2]
117	320	640	640	640	
122	320	640	320	160	
128	320	320	160	160	
Group II					
105	160	640	320	320	
116	160	320	320		N.T.
126	640	160	80	80	
217	320	640	320	160	
Group III					
101	320	640	640		N.T.
113	640	640	320	160	
123	320	160	640	640	
124	640	320	320	640	
Group IV					
102	640	640	640	640	
103	640	640	320		N.T.
114	320	320	640		320
115	320	160	320	640	
119	320	320	320	640	
120	640	640	640	640	

TABLE IV (Continued)

Calf No.	Jan. 29	Feb. 10	Feb. 23	Mar. 8	Mar. 16
121	320	640	320		320
125	640	640	640	640	

[1]Reciprocal of antibody dilution; all sera scored as 1+ degree of fluorescence.

[2]N.T. = Not Tested; Blank spaces indicate that calves were not bled that day.

and January 15), however, the calves still retained high titers (1:80) through March.

There was little variation noted between each group or between the transported and the nontransported calves. The only difference was that during the Pawhuska phase of the experiment, the transported calves reached titers of 1:640 quicker and retained this titer longer than did the nontransported calves. All 16 of the transported calves had a titer of 1:640 at four weeks, but only eight of nine (89 percent) of the control group still had a 1:640 titer. Also, 13 shipped calves (81 percent) still had a 1:640 titer at eight weeks (January 15), compared to five calves (56 percent) of the nontransported calves.

There also were no significant differences in antibody response between the groups in the Stillwater project. Calves aerosoled with BRSV or BRSV and Pasteurella multocida (groups III and IV) did not show marked increases in antibody titers, and some calves (103, 113 and 121) even showed a drop in titer. The groups not receiving BRSV (groups I and II) showed similar variation in antibody response.

Table V gives a description of the various CF antigens used and Tables VI, VII and VIII give the results obtained with these antigens. Despite the fact that several attempts and several modifications were made, all the BRSV antigens tested were either anticomplementary (AC) or gave low antigen titers.

The commercially prepared RSV antigen (Ag 1) gave the 1:8 titer specified by the manufacture when it was tested against their RSV antiserum. This antigen was then used to screen several bovine sera from the C-8 project. From this, two sera with the highest titers (referred to as bovine antiserum 114 and 115) were used in conjunction with the RSV antiserum to test the BRSV antigens.

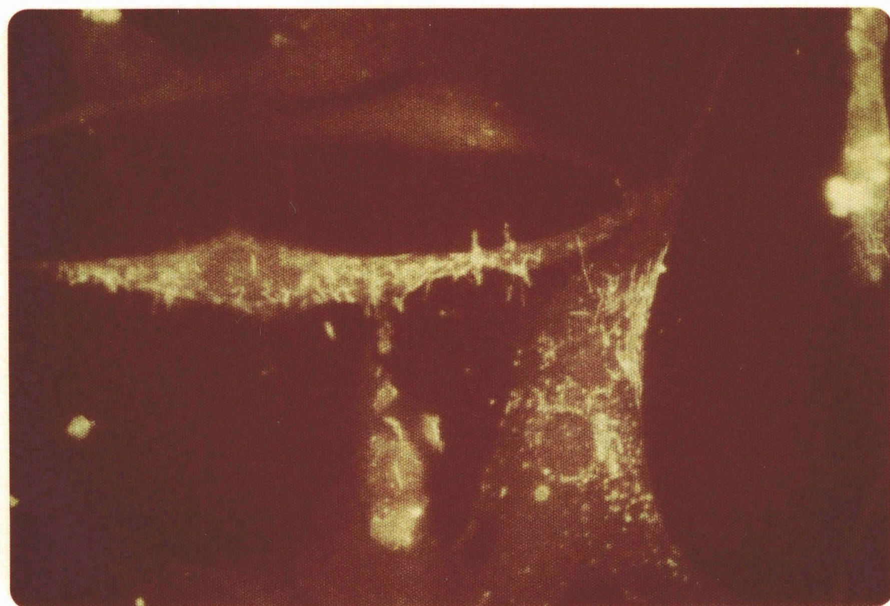

Figure 1. Specific Cytoplasmic Fluorescence of BRSV Antigen in GTU Cells. Mag. X312.5

TABLE V

DESCRIPTION OF VARIOUS PREPARATIONS OF CF ANTIGENS

Antigen	Description
Whole Culture	
Ag 1	Commercially prepared CF antigen to the Long strain of Human RSV (minimum titer of 1:8)
Ag 2	Antigen harvested at 75% or greater CPE
Ag 3	Antigen harvested at 75% or greater CPE
Ag 4	Medium from cells showing less than 25% CPE
Ag 5	Medium from same cells as Ag 4, but at 50% CPE
Ag 6	Medium from cells at 50% CPE
Ag 7	Same cells as Ag 6, but harvest of cells and medium at 75% or greater CPE
Ag 8	Medium from cells showing less than 25% CPE
Ag 9	Medium from cells showing less than 25% CPE
Ag 10	Medium from cells showing 40-60% CPE
Ag 11	Harvested medium and cells from Ag 10 at 75% or greater CPE
Ag 12	Ag 11 concentrated 10X with a millipore separator
Ag 13	Harvested medium and cells at 75% CPE, then concentrated 10X with a millipore separator
Ag 14	Dialysate portion of Ag 13
Ag 15	Antigen harvested at 75% or greater CPE
Ag 16	Concentrated cell phase from Ag 15; cells resuspended at 1/10 volume in R-saline
Ag 17	Fluid portion removed from Ag 16 when the cells were separated from the medium
Ag 101	Pooled antigen (produced the same as Ag 16)

TABLE VI

RESULTS OF WHOLE CULTURE OF ANTIGEN TITRATIONS

Antigen	Antiserum[1]	Titer[2]
Ag 1	RSV	1:8
Ag 2	RSV	1:4
Ag 2	115	-
Ag 3	RSV	1:4
Ag 3	115	AC
Ag 4	115	AC
Ag 5	115	AC
Ag 6	115	AC
Ag 7	115	AC
Ag 8	115	AC
Ag 9	115	-
Ag 10	RSV	-
Ag 11	RSV	1:2
Ag 12	115	1:2
Ag 12	RSV	1:2
Ag 12	RSV	AC
Ag 13	RSV	AC
Ag 14	RSV	-

[1]115 = Bovine serum from calf No. 115, C-8 project, serum drawn on March 8, 1976; a titer of 1:128 vs. antigen of Long strain of Human RSV. RSV = antiserum to the Long strain of respiratory syncytial virus; titer, 1:64.

[2]AC indicates the antigen is anticomplementary; (-) indicates negative at a 1:2 dilution.

TABLE VII

RESULTS OF CELL PHASE CF ANTIGEN TITRATIONS

Antigen	Antiserum[1]	Result
Ag 15	RSV	1:2
Ag 16	RSV	1:4
Ag 17	RSV	-
Ag 101	RSV	1:4
Ag 101	115	AC
Ag 101[2]	114	AC
Ag 101[3]	114	AC
Ag 101[4]	115	AC[5]

[1]Antiserum 114 from calf No. 114, C-8 project, March 16, 1976; a titer of 1:128 vs. antigen of Long strain of Human RSV.

[2]Antigen titration done using modified Kolmer saline diluent.

[3]Antigen titration done using LBCF veronal buffer diluent.

[4]Both Ag 101 and antiserum 115 were treated for anticomplementary activity before use.

[5]Both the antiserum and the antigen gave anticomplementary results.

TABLE VIII

COMPARISON OF TREATMENTS FOR ANTICOMPLEMENTARY ANTIGEN WITH DIFFERENT LEVELS OF COMPLEMENT MODIFIER

Ag 101 Treated With 10 Percent Guinea Pig Complement		
Percent Modifier	Antiserum	Titer
5	114	AC
5	RSV	--
10	114	AC
10	RSV	AC
--	114	--
--	RSV	1:4

Ag 101 Treated With 25 Percent Guinea Pig Complement		
Percent Modifier	Antiserum	Titer
5	114	AC
5	RSV	--
10	114	AC
10	RSV	AC
--	114	--
--	RSV	1:4

Whole culture antigens, Ag 2 and Ag 3, gave titers of 1:4 with RSV antiserum, but were negative and AC, respectfully, when tested with bovine antiserum 115. Antigens four through ten, harvested at various stages of CPE, were either AC or negative when tested with bovine antiserum 115 or RSV antiserum. Antigen 11 gave a 1:2 titer verse RSV antiserum then it was concentrated 10X by vacuum dialysis (Ag 12). However, this did not increase the titer with either the RSV or 115 antiserum, and it was AC when retested with RSV antiserum. Another antigen, Ag 13, was concentrated but it was also anticomplementary with RSV antiserum, however, the dialysate of this antigen, Ag 14, did not contain CF antigen.

When the whole culture antigen (Ag 15), cell phase antigen (Ag 16) and the culture medium removed from the cell phase antigen (Ag 17) were tested with RSV antiserum the titers were 1:2, 1:4 and negative, respectfully. This indicated that the CF antigen was only released after the cells were disrupted. When a pooled cell phase antigen (Ag 101) was tested with RSV antiserum, it again gave a 1:4 titer, but it was AC with both bovine antiserum 114 and 115.

Several attempts were made to find the cause of the AC activity. A different diluent, veronal buffer (24) was tried and the antigen and antiserum were treated for anticomplementary activity. However, none of these changes solved the problem.

An experiment was then done to test two types of AC treatment for antigens, using 0, 5, and 10 percent amounts of complement modifier (Table VIII). Results from this experiment showed there was no difference between the two AC treatments, but the test was AC only when the complement modifier was present.

Because of the problems encountered with BRSV antigens, attempts to use it were discontinued. Later it was decided to use the commercially prepared RSV antigen in the CF test. To overcome the AC activity of the complement modifier, the modifier was added to the complement diluent (at a five percent concentration) to perform the complement titration. This proved to be an effective method of eliminating the AC problems encountered previously.

The sera from seven calves from the C-8 project, collected at four to six week intervals, were selected for the CF test. The antibody titers obtained with these sera are given in Table IX. All of the calves were negative at the beginning of the experiment, then showed a rapid rise to their highest titer at four weeks. The titers began to decrease after this but all the calves still had a 1:8 or greater titer in March. Calf 115, however, reached his highest titer both at four weeks and at sixteen weeks (March 8).

Four of these calves had been transported during the Pawhuska experiment (calves 102, 115, 120 and 123) and three of them (102, 115 and 120) had higher titers than the nontransported calves at four weeks. Two of the control (105 and 127) reached a high titer of 1:32 and the third control calf (117) reached a high titer of 1:64 in four weeks.

Also, four of the calves (102, 115, 120 and 123) were aerosoled with BRSV shortly after the January 29 bleeding, three of which showed a rise in titer or a steady titer five weeks later (March 8). The fourth calf (102), although not showing an antibody rise did retain a significant titer of 1:32 on March 8. Meanwhile, those calves not receiving BRSV (105, 117 and 127) showed steadily decreasing titers during the Stillwater project.

TABLE IX

COMPLEMENT FIXATION ANTIBODY TITERS FROM THE C-8 PROJECT

Calf No.	Nov. 13	Dec. 11	Jan. 29	Mar. 8
102	-[2]	256	128	32
105	-	32	16	8
115	-	128	8	128
117	-	64	32	8
120	-	128	32	64
123	-	64	16	16
127	-	32	16	8

Treatments

Calf No.	Pawhuska Phase (Nov. 13 - Dec. 11)	Calf. No.	Stillwater Phase (Jan. 29 - Mar. 8)
102	PBS, transported	102	BRSV and *Pasteurella multocida*
105	PBS	105	PBS
115	PBS, transported	115	BRSV and *Pasteurella multocida*
117	PBS	117	*Pasteurella multocida*
120	Pasteurella Bacterin, transported	120	BRSV and *Pasteurella multocida*
123	Pasteurella Bacterin, transported	123	BRSV
127	Pasteurella Bacterin	127	PBS

[1]All sera were tested using four units of CF antigen to the Long strain of Human RSV.
[2]Titers are the reciprocal of the antiserum dilution: negative (-) indicates a titer of less than 1:8.

These results are in general agreement with the IFA titers, with the highest titers (or intensities of fluorescence) reached in four weeks, then the titers or fluorescence began to decrease. Significant titers were still evident, though, after sixteen weeks. The CF test was not as sensitive as the IFA test. It did not detect the positive serum (calf 127) or the borderline reaction (calf 105) on November 13, and the CF titers were considerably lower than the IFA titers. However, since the CF test was done using RSV antigen, these titers are probably lower than what would be found using a suitable BRSV antigen.

CHAPTER V

DISCUSSION

The purpose of the virus titration experiments was to determine the usefulness of the IFA technique for titrating BRSV. To do this the IFA method was compared to a MG-G titration method. After several modifications were tried, it was found that under certain conditions the IFA method was as sensitive as the MG-G method, but required a shorter incubation period.

Thirty hours incubation was chosen for the IFA procedure because this was thought to be the time of maximum fluorescence, and five to seven days is required for the MG-G method for complete development of CPE. However, the 30 hour incubation period was not the time of maximum fluorescence, since it was less sensitive than the MG-G method. This was true even when the virus inoculum was increased from 0.1 ml to 0.25 ml.

Because of this problem, attempts were made to increase the incubation time for the IFA procedure. It was found that fluorescence could be detected at 30, 48, 96 and 120 hours, which was not in agreement with previous research findings. Potgieter was able to detect slight fluorescence at 72 hours incubation, but no fluorescence at 96 hours (9). Based on these findings, IFA titrations were incubated for 72 and 120 hours. At these longer incubation periods, the IFA method was as sensitive or more sensitive than the MG-G titration method, but

the IFA titers could be determined two days earlier.

The lower IFA titers at 30 hours incubation was not expected, since this is sufficient time to produce fluorescence when a larger inoculum is used. Random variation between the IFA and MG-G stained slides may have accounted for some or all of this difference. It is also possible that fluorescing cells were so few that they were not observed, or it may be that a larger virus inoculum is required to produce maximum fluorescence at 30 hours.

The MG-G and IFA methods require about the same amount of time to perform and both are tedious, time consuming procedures requiring close examination of the cells. However, since an IFA method may give slightly higher titers and requires a shorter incubation period, this method has an added advantage over the MG-G method.

Another use of the IFA technique is in determining serum antibody titers. In the present study, when it was used in conjunction with microtiter tissue culture plates, it was found to be a rapid, inexpensive method of determining antibody titers from a large number of samples.

The C-8 project also offered an opportunity to apply the IFA technique to the diagnosis of BRSV infections. The results indicated that antibody titers may rise rapidly and high titers of 1:640 or greater are not uncommon. Also these high titers may be detected several months after the initial infection. This information may be useful in determining whether the antibodies are of natural or maternal origin and in determining the exact time of infection.

Some researchers have suggested that calves may not become infected from their dams, but are infected after being commingled with different

groups of calves (18). Since these calves represent a closed herd, they must have been infected from their dams. Also, since most of the calves were negative at the beginning of the experiment and all the calves showed a rise in titer, their antibodies must have been of natural origin.

Why there was a rapid increase in antibodies during the first week of the experiment is uncertain. It is possible the virus was present in too low of a concentration to stimulate serum antibody production, but with the stress due to transportation and treatment, virus titers increased enough to stimulate serum antibody production. Another explanation may be that the IFA test is so sensitive that a moderate increase in antibody levels may mean a sharp increase in IFA antibody titers. This may also explain why antibody titers remained at such high levels throughout the experiment.

The lack of significant difference between the four groups of the Pawhuska project may have been due to the design of the project. Since the calves were pastured together before and during the experiment, the virus had ample opportunity to spread through the herd. One difference that was observed was that most of the transported calves reached their highest titers sooner and retained these titers longer than the non-transported calves. This may indicate that the stress of transportation affected the antibody response of the calves.

There also was little difference noted between the groups of the Stillwater project, despite the fact that two of the groups (groups three and four) were aerosoled with bovine RS virus. A secondary response may not have occurred because of too low of a virus dosage, or the secondary response may not have included serum IgG antibodies.

The use of microtiter tissue culture plates was also found to be a very useful technique. Because of their decreased size, they accounted for great savings in tissue culture cells, medium and reagents, and the plates were easier to handle than the equivalent number of chamber/slides.

Using microtiter plates does require some precautions. First of all, during fixation the acetone must be added carefully to each well or the wells may be etched enough that the cells cannot be seen.

Another problem is that a special long working distance objective is needed for the fluorescent microscope. Because such objectives are expensive, this generally means interpretations must be made with a single objective. These special objectives are also a low magnification, such as X20, as the one used in the present study. Although this is adequate for scanning, it does create some problems in distinguishing between nonspecific and specific staining. This problem was most apparent at low serum dilutions (or undiluted serum) and at high dilutions near to endpoints. Because of this, the useful range of serum dilutions in the present study was found to be between 1:10 and 1:640. When comparisons were made between chamber/slides and microtiter plates, the microtiter plate method gave similar results to the chamber/slide method within this range of serum dilutions.

Most of the work with the CF test was disappointing, mainly because a suitable antigen to BRSV could not be produced in cell cultures. The whole culture antigen, which is used for human RS virus, never gave high enough titers to be useful. Further research indicated that the CF antigens were intracellular, so efforts were focused on using a cell phase antigen.

Although the cell phase antigen gave better results, it was anti-complementary when used with bovine antiserum. It was later decided that at least part of this problem was caused by the complement modifier. Despite the AC problem, antigen titers of 1:8 or greater did not appear to be possible. Since there did not appear to be a solution for these problems, work with BRSV antigen was discontinued. Further research with cell phase, though, may show this to be an acceptable antigen.

Since low antigen titers were obtained with both types of antigen using RSV antserum, some antigen must have been present. According to previous reports, these titers should have been higher using bovine antiserum (1,15). Several things may have caused the problems with BRSV antigens. The strain of virus, cell line, culture medium, size of inoculum, length of incubation and the choice of bovine antiserum may all have had an adverse effect on the results.

Japanese researchers immunized rabbits with BRSV and used their sera to determine antigen titers. This is probably a better approach, since antibodies to a specific strain of virus are obtained, and the complement modifier is not needed with rabbit serum. They also used a different strain of virus, cell line and medium, which may explain why they could use whole culture antigen (15).

The CF test, though not as sensitive as the IFA test still indicated high titers and titers of 1:8 or greater were still apparent sixteen weeks later. The antibody response was more what was originally expected in that high titers were reached in about four weeks then titers began a steady decline. Also the four calves that were aerosoled with BRSV showed more of a secondary antibody response than those calves not receiving BRSV. The calves that received BRSV either had a steady

titer or an increased titer about five weeks after receiving the virus. From this it appears that the CF test was more suitable in detecting the secondary response, therefore it may be a better test for detecting reinfections than the IFA test.

The CF test, using RSV antigen, appears to be a useful diagnostic tool, especially for those laboratories already familiar with the CF test. No one has yet done an extensive comparison of bovine antibody titers obtained using both RSV antigen and BRSV antigen. The antibody titers obtained using RSV antigens are probably one or two dilutions lower than they would be using BRSV antigen. Since RSV antigens are available commercially, thus eliminating the need for cell cultures, using RSV antigen may be more practical than using BRSV antigens.

An advantage of the CF test is that it does not require the specialized and expensive equipment needed for the IFA test. It also has its disadvantages. The CF test requires a great deal of experience in order to get consistent results, and since exact volumes and titers must be determined it is more subject to technical errors. Other problems are that some sera are anticomplementary even after treatment, and that a suitable complement modifier is needed to test bovine serum.

CHAPTER VI

SUMMARY

An IFA method was developed for determining BRSV titers. When this method was compared to the MG-G method, it was found to be as sensitive as the MG-G method but titers could be determined 48 hours earlier using the IFA method.

Goat turbinate cells, infected with BRSV, were grown in microtiter tissue culture plates and were stained and examined by an IFA technique. This method was used to determine the antibody titers of 25 calves during a four month period. Antibody titers increased rapidly and titers of 1:80 or greater were still present almost four months after the initial infection. This rapid antibody response in this age group of calves may be significant in determining whether antibodies are of maternal or natural origin and the prolonged high titers may create problems in determining the exact time of infection.

Attempts to produce CF antigens to the FS1-1 strain of BRSV were unsuccessful. Commercially prepared antigens to the Long strain of human RSV were available and were used to test sera from seven of the calves. The CF test was not as sensitive as the IFA method, but antibodies were still detectable at the end of four months. However, the CF test appeared to detect a secondary antibody response from four of the calves, five weeks after they had been aerosoled with bovine RS virus. A secondary response was not apparent from the IFA test results.

LITERATURE CITED

(1) Paccaud, M. F. and C. L. Jaquier. "A Respiratory Syncytial Virus of Bovine Origin." Arch. Gesamte Virusforch., Vol. 30 (1970), 327-342.

(2) Inaba, Y., Y. Tanaka, K. Sato, T. Omori, and M. Matumoto. "Bovine Respiratory Syncytial Virus, Studies of an Outbreak in Japan, 1968-1969." Japan J. Microbiol., Vol. 16 (5) (1972), 373-383.

(3) Wellemans, G., J. Leunen, and E. Luchsinger. "Respiratory Ailments of Cattle: Isolation of a Virus (220/69) with a Serological Resemblence to Human Respiratory Syncytial Virus." Ann. Med. Vet., Vol. 114 (1970), 89-93.

(4) Jacobs, J. W. and N. Edington. "Isolation of Respiratory Syncytial Virus From Cattle in Britain." Vet. Rec. Vol. 88 (1974), 694.

(5) Smith, M. H. "Isolation, Characterization and Pathogencity Studies of Bovine Respiratory Syncytial Virus." (Unpub. Ph.D. dissertation, Iowa State University, 1974).

(6) Rosenquist, B. D. "Isolation of Respiratory Syncytial Virus From Calves with Acute Respiratory Disease." J. Inf. Dis., Vol. 130 (2) (1974), 1977-182.

(7) Rossi, C. R. and G. K. Kiesel. "Serological Evidence for the Association of Bovine Respiratory Syncytial Virus with Respiratory Tract Disease in Alabama Cattle." Inf. and Imm., Vol. 10 (2) (1974), 293-298.

(8) Berthiaume, L., J. Joncas, G. Boulay and V. Pavilanes. "Serological Evidence of Respiratory Syncytial Virus Infection in Sheep." Vet. Rec. (1973), 337-338.

(9) Potgieter, L. N. D. and P. L. Aldridge. "The Use of Indirect Fluorescent Antibody Test for Studying Bovine Respiratory Syncytial Virus." 57th Annual Meeting of the Conference of Research Workers in Animal Disease. Abst. 63, 1976.

(10) Beem, M. and D. Hamre. "Respiratory Syncytial Virus." Diagnostic Procedures for Viral and Rickettsial Infections. 4th Ed. Ed. E. H. Lennette. New York: American Public Health Association Inc., 1969, 491-503.

(11) Doggett, J. E., D. Taylor-Robinson, and R. G. C. Gallop. " A Study of an Inhibitor in Bovine Serum Active Against Respiratory Syncytial Virus." Arch. Gesamte Virusforch., Vol. 23 (1968), 126-137.

(12) Inaba, Y., Y. Tanaka, T. Omori, and M. Matumoto. "Physicochemical Properties of Bovine Respiratory Syncytial Virus." Japan J. Microbiol., Vol. 17 (3) (1973), 211-216.

(13) Wellemans, G. and J. Leunen. "The Role of V220/69 (Bovine Respiratory Syncytial Virus) in Respiratory Disorders of Belgium Cattle." Ann. Med. Vet., Vol. 115 (1971), 35-39.

(14) Wellemans, G. and J. Leunen. "Respiratory Syncytial Virus and Respiratory Disorders in Cattle." Ann. Med. Vet., Vol. 119 (6) (1975), 359-369.

(15) Takahashi, E., Y. Inaba, H. Kurogi, K. Sato, Y. Goto, and T. Omori. "Diagnosis of Bovine Respiratory Syncytial Virus by Complement Fixation." Nat. Inst. Anim. 11th Quart., Vol. 15 (1975), 179-185.

(16) Rinaldini, L. M. "An Improved Method for the Isolation and Quantitative Cultivation of Embryonic Cells." Exp. Cell. Res., Vol. 16 (1959), 477-505.

(17) Reed, L. J. and H. Muench. "A Simple Method of Estimating Fifty Per Cent Endpoints." Am. J. Hyg., Vol. 27 (1938) 493-497.

(18) Potgieter, L. N. D. and P. L. Aldridge. "Use of the Indirect Fluorescent Antibody Test in Detection of Bovine Respiratory Syncytial Virus Antibodies in Bovine Serum." J. Vet. Res., Vol. 38 (9) (1977), 1341-1343.

(19) Pursell, A. R. and J. R. Cole. "Procedure for Fluorescent Antibody Staining of Virus Infected Cells in Plastic Plates." J. Clin. Micro., Vol. 3 (1976), 537-540.

(20) Schmidt, N. J. and E. H. Lennette. "Comparison of Various Methods for the Preparation of Viral Serological Antigens From Infected Cell Cultures." Appl. Micro. Vol. 21(2) (1971), 217-226.

(21) Lennette, E. H. "General Principles Underlying Laboratory Diagnosis of Viral and Rickettsial Infections." Diagnostic Procedures for Viral and Rickettsial Infections. 4th Ed. Ed. E. H. Lennette. New York: American Public Health Association Inc., 1969, 52-58.

(22) Von Zeipel, G. "Prevention and Removal of Anticomplementary Activity of Tissue Culture Fluids." Arch. Gesamte. Virusforsch., Vol. 8 (1958), 246-258.

(23) Lennette, E. H. "General Principles Underlying Laboratory Diagnosis of Viral and Rickettsial Infections." Diagnostic Procedures for Viral and Rickettsial Infections. 4th Ed. Ed. E. H. Lennette. New York: American Public Health Association Inc., 1969, 37-38.

(24) U.S. Public Health Service. Standardized Diagnostic Complement Fixation Method and Adaption to Micro Test. Publ. No. 1228. Washington: U.S. Public Health Service, 1964.

APPENDIX

THE C-8 BACTERIN EXPERIMENT

The serum samples tested were from the 25 calves used in the C-8 bacterin experiment that was conducted by the Respiratory Disease Laboratory. These calves were raised at the Veterinary Research Station at Pawhuska, Oklahoma, and were recently weaned calves weighing 400-500 pounds. The experiment was started November 12, 1975 and ended when the calves were slaughtered on March 8 and March 15, 1976.

The experiment was divided into two phases. The objectives of the Pawhuska phase were to study the toxicity of *Pasteurella multocida* and *Pasteurella hemolytica* and to evaluate different methods of infecting calves with these organisms. The calves were divided into four groups, and some calves from each group were transported in an open truck while the remaining calves served as non-transported controls (Table X). Calves to be transported were loaded on an open truck and transported for 12 continuous hours, then returned to the station. The initial collection of nasal, tracheal and serum specimens was done at this time then the calves were given the treatments shown in Table X. All the calves were maintained together on open pasture at the experimental station. Specimens were again collected and the treatments repeated on November 20, 1975. Additional specimens were collected at weekly intervals throughout the experiment.

The second phase of the experiment was started by transporting 20

of these calves to the Veterinary Science area at Stillwater. The objectives of this phase were to study what effect bovine respiratory syncytial virus has, alone or in combination with *Pasteurella multocida*, in bovine respiratory disease.

The calves were again divided into four groups and given the treatments shown in Table XI. Each group was kept at separate locations in the Veterinary Science area. Serum, nasal and tracheal specimens were collected at weekly intervals until 14 of the calves were slaughtered on March 8, and the other six calves were slaughtered on March 15, 1976.

TABLE X

TREATMENTS GIVEN CALVES IN PAWHUSKA PROJECT

Calf No.	Group No.	Trucked or Control	Treatment[1]
101-103	1	Trucked	10 ml of saline subcutaneous
104-105	1	Control	
107-109	2	Trucked	5 ml 5964 Bacterin and 5 ml 6013 Bacterin,
110-111	2	Control	subcutaneous
113-115	3	Trucked	10 ml of saline by aerosol
116-117	3	Control	
119-125	4	Trucked	5 ml 5964 Bacterin and 5 ml 6013 Bacterin by aerosol
126-128	4	Control	

[1]Bacterin 5964, Pasteurella multocida; Bacterin 6013, Pasteurella hemolytica.

Treatment Schedule: 11-12-75 - Calves transported for 12 hours. 11-13-75 - Calves given initial treatments. 11-20-75 - Treatments were repeated.

TABLE XI

TREATMENTS GIVEN CALVES IN STILLWATER PROJECT

Calf No.	Group No.	Treatments
104, 117, 122, 128	1	Aerosoled with *Pasteurella multocida*
105, 116, 126, 127	2	Aerosoled with phosphate buffered saline (PBS)
101, 113, 123, 124	3	Aerosoled with Bovine RS virus
102, 103, 114, 115, 119, 120, 121, 125	4	Aerosoled with Bovine RS virus and *Pasteurella multocida*

Treatment Schedule:

1-30-76 Aerosoled groups 1 and 2 with *P. multocida*.

2-2-76 Aerosoled groups 3 and 4 with bovine RS virus.

2-9-76 Aerosoled groups 2 and 4 with PBS: aerosoled groups 1 and 4 with *P. multocida*.

VITA 2

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