Experimental Infection of Calves with Bovine Respiratory Syncytial Virus (Quebec Strain)

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ABSTRACT

An experiment was designed to evaluate the clinical, haematological, viral and serological aspects of bovine respiratory syncytial virus infection in calves. Eleven calves were inoculated intranasally with bovine respiratory syncytial virus (Quebec strain) in aerosol. Clinical, haematological and serological responses of the calves and virus shedding were monitored.

The experimentally infected animals manifested moderate to severe signs of respiratory disease. The parameters used to evaluate the severity of the disease included ocular discharge, conjunctivitis, lung sounds, nasal discharge, pyrexia and leukopenia. The animals were scored accordingly (scale infected 70.8-148.5, control 22-29.3). Highest disease scores were observed between day 6-9 after infection. Virological and serological assessment demonstrated that the observed clinical picture was due to bovine respiratory syncytial virus infection.

RÉSUMÉ

Cette expérience consistait à étudier les aspects cliniques, hématologiques, virologiques et sérologiques de l'infection de veaux par le virus syncytial respiratoire bovin. Les auteurs en inoculèrent à cette fin 11, au moyen d'aérosols d'une souche québécoise de ce virus. Ils vérifièrent ensuite les réactions cliniques, hématologiques et sérologiques, ainsi que l'excrétion de virus.

Certains veaux expérimentaux manifestèrent des signes respiratoires modérés et d'autres, des signes plus marqués. Les paramètres qui servirent à déterminer la gravité de l'infection incluaient: l'écoulement oculaire, la conjonctivite, les râles pulmonaires, l'écoulement nasal, l'hyperthermie et la leucopénie. D'après l'échelle de ces critères, les veaux expérimentaux se situaient entre 70,8 et 148,5, tandis que les témoins se situaient entre 22 et 29,3. La maladie atteignit un sommet, de six à neuf jours après l'infection. Les épreuves virologiques et sérologiques démontrèrent que les signes cliniques manifestés par les veaux expérimentaux résultaient d'une infection par le virus syncytial respiratoire bovin.

INTRODUCTION

A new agent designated bovine respiratory syncytial virus (BRSV) was isolated from the respiratory tract of cattle in association with a respiratory disease by Inaba *et al* (7). Evidence of this association was supported by the demonstration of antibody against human respiratory syncytial virus (HRSV) in bovine serum, suggesting that a virus antigenically similar to HRSV was present in cattle. Since this early work, serological detection and isolation of the BRSV from the respiratory tract during epizootic outbreaks of respiratory disease have been reported (7, 8, 11, 14, 17, 18, 20).

Although this virus represents a possible etiological agent involved in the pathogenesis of the respiratory disease complex, the intensity and the onset of the clinical signs reported have been largely contradictory (10, 15, 21). Inaba *et al* (7) demonstrated a severe disease in a natural infection with mortality of 0.025% and Wellemans and Leunen (21) reported an

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influenza like disease with sudden mortality due to acute pulmonary emphysema. On the other hand Smith *et al* (18) did not observe any mortality in an experimental study of the BRSV infection.

Thomas *et al* (19) suggested that discrepancies between the findings were probably due to the method of computing the clinical signs. They suggested a method of disease assessment and used it to evaluate the clinical response of calves infected with bovine parainfluenza type 3 (PI3) virus or with a combination of BRSV and bovine viral diarrhea virus (BVD).

This experiment was designed to evaluate the clinical, haematological, viral and serological response of calves infected with a BRSV (designated Q-1).

MATERIALS AND METHODS

CELL CULTURES

Primary bovine fetal skin cells (BFS) and Madin Darby bovine kidney cell line (MDBK) were grown in Eagle's minimum essential medium with Earle's base (EMEM) containing 10% fetal calf serum (FCS)[1] and were maintained in EMEM with 5% horse serum[1]. To both media 100 IU/mL penicillin and 100 μg/mL streptomycin were added.

VIRUS CHARACTERIZATION

Bovine respiratory syncytial virus (Q-1) was isolated from the nasal secretion of a Holstein calf on BFS cell cultures as described under virus isolation. The stock virus at the third passage was produced in BFS cell cultures. The type of nucleic acid, chloroform and pH sensitivities of the virus were determined according to Feldman and Wang (2) and Hamparian *et* al (4). Hemadsorption test was conducted as described (5) using bovine erythrocytes.

Final identification of the isolated virus was made by electron microscopic (EM) examination of negatively stained tissue culture fluid (12) and by the indirect fluorescent antibody test (FA) using a BRSV (FSL-1 strain) antiserum as described under serology. The specificity of BRSV antiserum was determined in a preliminary study which showed that this serum was not able to inhibit the cytopathic effect (CPE) of BVD or infectious bovine rhinotracheitis virus (IBR) in the neutralization test.

ANIMALS

Fourteen one to five week old Holstein calves were used in this experiment. These calves were free of IBR, BVD, P13, BRSV, Reo and bovine adenovirus type 3 and of antibodies to these viruses.

DESIGN OF EXPERIMENT

The calves were housed in eight separate rooms, one or two calves per room. Eleven calves in seven groups were each exposed intranasally to 2 x 10^6 median tissue culture doses ($TCID_{50}$) of BRSV (Q-1) in 10 mL of EMEM. The inoculum was delivered in aerosol from a nebulizer adjusted to give particle size of 0.5 to 3 μm at 60% relative humidity. Three control animals housed separately were treated identically with EMEM.

A daily clinical assessment was performed during the eight days preceeding the experimental inoculation and twice a day during the experimental period. Examinations were made at approximately the same time each day. Starting with controls, the animals were examined in the same order by the same people.

Clinical signs and their scores were recorded for 20 days (eight pre and 12 post-inoculation days). Clinical and haematological scores were analysed as described (19) with the exception that "wasting" was replaced by "lung sounds", respiratory rate was estimated for one minute and cough was assessed by either external palpation or by direct observation during clinical examination.

HAEMOTOLOGICAL INVESTIGATION

Blood samples were taken in heparinized vacutainer tubes at three day intervals for haematological studies. Erythrocyte, leukocyte, hematocrit, haemoglobin and mean cell values were studied as described (19). Scores for each haematological parameter were calculated for 12 days after inoculation and statistically analysed.

[1]Grand Island Biological Company, Grand Island, N.Y.

VIRUS ISOLATION

Nasal secretion from control and infected calves were collected one day before infection and then each day after infection, starting with the second day and continuing up to the 12th day. Within two hours of collection, the samples were diluted 1:4 in Hank's balanced salt solution (HBSS) containing 1000 IU/mL penicillin, 1000 μg/mL streptomycin and 2 μg/mL fungizone. The obtained suspensions were centrifuged at 600 g for 10 min, after which 0.5 mL of the supernatant was absorbed for one hour on each of four monolayer tube cultures of BFS cells. The remaining portions of the supernatant were stored in liquid nitrogen for titration. The cell cultures were washed with EMEM supplemented with 2% horse serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin, and incubated in the medium at 37°C for seven days. Cell cultures were examined each day for the presence of cytopathic effect (CPE). Negative cultures were passaged twice in FBS cells.

VIRUS TITRATION

Positive nasal secretion samples were titrated in BFS cells on Labtek chamber slides as described (1). The cultures were stained by haematoxylin and eosin and examined for CPE. Detection of virus by its CPE in stained cell cultures was much more sensitive than detection of the virus in unstained cultures. The infectivity titer of the sample was expressed in $\log_{10}$ $TCID_{50}$/1 mL and was calculated using the Spearman-Kaerber procedure.

SEROLOGY

All sera were heat inactivated at 56°C for 30 min before use. Antibody titer to IBR virus (Cooper strain), BVD virus (NADC-36) and bovine adenovirus type 3 (BAV-3) (WBR-1 strain) were determined using neutralization test (6) performed in microtiter plates. All serum samples were tested in triplicates using twofold dilutions with 0.05 mL of 100 $TCID_{50}$ of the virus added to an equal volume of the serum. The mixtures were incubated in the microtiter plates for one hour at room temperature. Madin Darby bovine kidney or BFS cells were prepared in EMEM, to contain 160,000 cells per mL. To each well, 0.1 mL of the cell suspension was added and the plates were incubated at 37°C in a CO_2 incubator. The results were read after five days or three days for BVD.

Antibody to bovine parainfluenza type 3 (PI-3) were detected by the haemagglutination inhibition test in microtiter plates using a 0.5% suspension of bovine erythrocytes in saline and four haemagglutinating units of PI-3 antigen (6).

To demonstrate the presence of antibodies to BRSV, 50% confluent monolayer cells of bovine fetal skin were used. The cells were grown on Labtek slides in EMEM supplemented with 5% horse serum and infected with the FSL-strain of BRSV, each well receiving 10^3 $TCID_{50}$. After 35 h incubation at 37°C in a CO_2 incubator, the infected cultures were rinsed in phosphate buffered saline (PBS) and fixed in acetone for 10 min. The fixed slides were stored at −70°C until used. The indirect fluorescent antibody test was carried out on the slides according to Potgieter and Aldridge (13). Briefly the infected cells were covered with serum and incubated for 30 min at 37°C in a moist chamber. After washing three times for 20 min, the slides were rinsed briefly in distilled water and allowed to dry at room temperature. The infected cells were covered with fluorescein conjugated rabbit antibovine immunoglobulin[2] diluted 1:64 in PBS, and incubated and washed as before. Cover slips were mounted and the preparations were examined with a fluorescent microscope. The fluorescence in BRSV infected cells appears as a granular cytoplasmic fluorescence. Controls included BRSV infected and noninfected cells treated with fetal calf serum and specific antisera to IBR and BVD viruses.

The reovirus antibody was determined by complement fixation (CF) test in microtiter plates (16) using reovirus group specific antigen[3].

RESULTS

VIRUS CHARACTERIZATION

Bovine fetal skin cells infected with

[2]Miles Laboratories, Elkhart, Indiana.

[3]Microbiological Associates, Bethesda, Maryland.

BRSV (Q-1) did not hemadsorb bovine red blood cells. The virus was sensitive to lipid solvent, labile at low pH, sensitive to heat and considered to be a ribonucleic acid containing virus. Delicate fluorescent granular networks were observed in the cytoplasm of the BFS cell culture infected with Q-1 or SFI-1 strains of BRSV when stained with BRSV antiserum (SFI-1). No fluorescence was observed when BVD antiserum or negative bovine serum were used.

Highly pleomorphic virions with spike projections were observed in the EM. Free nucleocapsids were seen as flexible filamentous loosely arranged helices with usual diameter of 15 nm.

CLINICAL ASSESSMENT

Disease scores from infected animals varied from 70.5 to 148.5 (Table I). The response was classified as moderate in two animals (< 100) and severe in the other nine animals (> 100), particularly for the period from six to nine days postinfection. The disease scores for controls were always subclinical (< 30) during the experiment. Nasal discharge, conjunctivitis and ocular discharge appeared by day 3, persisted to day 9, and decreased to about normal by day 12.

A significant difference in overall mean temperature ($P < 0.01$) (Fig. 1) was observed between the control and the infected calves. The infected calves showed a temperature increase on day 2 which persisted until day 6, thereafter the temperature gradually decreased.

TABLE I. Disease Scores of Control and BRSV (Q-1) Infected Calves

Pen number	Animal state	Calf number	Disease scores[a]
1	Infected	1	101.4
		2	121.9
2	Infected	3	143.2
		4	148.5
3	Infected	5	119.9
4	Infected	6	90.9
		7	70.5
5	Infected	8	108.1
		9	107.4
6	Infected	10	101.1
		11	109.1
7	Control	12	22.0
		13	29.3
8	Control	14	25.2

[a]Subclinical < 30
Moderate 51–100
Severe < 100

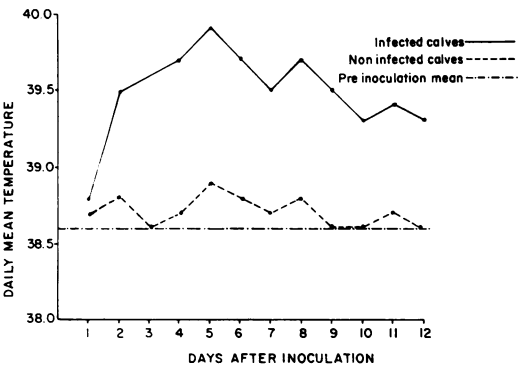

Fig. 1. Mean temperature of calves infected with BRSV (Q-1) compared to that of noninfected controls (standard deviation = 0.07).

The haematological parameters studied in the experiment demonstrated significant differences between control and infected calves. The leukocyte count (Table II), decreased on day 3 in the infected calves and remained low for seven days.

VIRUS RECOVERY

Virus recovery and titers from experimentally infected and control calves are given in Table III. Generally the inoculated calves shed BRSV in their nasal secretion from one to 12 days postinoculation with the highest virus titers detected between day 4 to 6.

SEROLOGICAL RESPONSE

Bovine respiratory syncytial virus antibody titers, as determined by the indirect fluorescent antibody technique are shown in Table III. The infected calves began to respond serologically to BRSV at six days postinoculation. The highest antibody titer was 1:128. The control calves were seronegative for the BRSV throughout the experiment.

DISCUSSION

In previous studies, BRSV produced mild to severe clinical signs of respiratory disease in experimental or natural infection (7, 15, 18, 21). In this experiment BRSV (Q-1) produced a severe respiratory syndrome in most infected calves.

The clinical signs observed in our experi-

TABLE II. Comparison of Average Haematological Values in Control and BRSV (Q–1) Virus Infected Calves

Animal groups	Haematological parameter	Preinoc.[a] mean	Postinoc. mean	Postinoc. S.D.
Noninfected	Erythrocytes		7.70	
Infected	(x 10^6)	7.77	8.27	1.68
Noninfected	Leukocytes		10.99[b]	
Infected	(x 10^3)	8.98[b]	7.56[b]	1.56
Noninfected	Mean cell values		41.30	
Infected	(Cu^{μ})	41.81	42.90	1.80
Noninfected	Hematocrit		34.60[b]	
Infected	(%)	33.12[b]	30.40[b]	0.30
Noninfected	Haemoglobin		10.90	
Infected	(g/100 mL)	10.48	11.90	2.30

[a]Preinoculation mean of the 14 calves
[b]Significant difference $P < 0.05$

TABLE III. Virus Titers and Serum Antibody Response in Calves Experimentally Infected with BRSV (Q–1)

Animal		Days postinoculation									
		Virus titers[a]					FA titers				
Number	State	1	4	6	9	12	1	4	6	9	12
1	Infected	0.5	1.5	2.2	1.5	0.5	—	—	16	128	128
2	Infected	—	2.0	2.0	1.0	—	—	—	4	16	16
3	Infected	—	1.0	3.8	0.0	—	—	—	4	16	16
4	Infected	—	0.5	1.5	0.5	—	—	—	8	32	64
5	Infected	2.0	3.5	4.0	2.0	—	—	—	4	64	64
6	Infected	0.5	1.0	0.5	—	—	—	—	8	32	64
7	Infected	1.5	—	1.3	—	—	—	—	16	128	32
8	Infected	1.5	1.0	3.5	3.2	1.0	—	—	4	16	32
9	Infected	0.5	0.5	2.0	2.3	—	—	—	32	32	16
10	Infected	—	1.0	3.2	1.0	0.5	—	—	8	16	32
11	Infected	—	0.5	2.3	1.8	1.0	—	—	4	16	64
12	Control	—	—	—	—	—	—	—	—	—	—
13	Control	—	—	—	—	—	—	—	—	—	—
14	Control	—	—	—	—	—	—	—	—	—	—

[a]Log_{10} $TCID_{50}$/1 mL

ment agreed with previous reports that pyrexia, leukopenia and nasal discharge were important clinical signs of the disease but failed to show that coughing or anorexia were significant. Lung sounds, conjunctivitis and ocular discharge have not been previously reported.

Several workers have analysed statistically temperature, haematological values and preinoculation clinical base lines of individual animals (3, 9, 19) without which the significant variables in our studies could not have been detected. The decision to limit the period of disease monitoring to 12 days postinfection, was made on the basis of the observation made by Thomas *et al* (19) and Smith *et al* (18) who found that the period of viral shedding coincided with the period of disease response by the animal. In our study, the highest disease scores were observed between days 6-9 and the highest virus titers were detected on day 6 postinfection. However, the virus was detected in small to moderate quantities throughout the observation period in most animals. The lower titers of virus recovered from some calves (Table III) could explain the moderate disease response of these animals. Leukocytosis and higher hematocrit observed in the noninfected (controls) animals were due to a mild diarrhea which lasted for two days during the experiment.

Recovery of BRSV and the presence of

antibody against this virus confirmed that the clinical signs observed were due to BRSV (Q-1).

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REFERENCES

1. **ELAZHARY, M. A. S. Y.** Aerosol stability of some bovine respiratory viruses. Ph.D. Thesis. University of Guelph, Guelph, Ontario. 1977.
2. **FELDMAN, H. A. and S. S. WANG.** Sensitivity of various viruses to chloroform. Proc. Soc. exp. Biol. Med. 106: 736-738. 1961.
3. **FRANK, G. H. and R. G. MARSHALL.** Relationship of serum and nasal secretion-neutralizing antibodies in protection of calves against parainfluenza-3 virus. Am. J. vet. Res. 32: 1707-1713. 1971.
4. **HAMPARIAN, V. V., M. R. HILLEMAN and A. KETLER.** Contributions to characterization and classification of animal viruses. Proc. Soc. exp. Biol. Med. 112: 1040-1050. 163.
5. **HOTCHIN, J. E., R. BEIBEL, L. M. BENSON.** Location of non-cytopathic myxovirus plaques by hemadsorption. Virology 10: 275-280. 1960.
6. **HSIUNG, G. D.** Diagnostic Virology. pp. 20, 41 and 46. New Haven and London: Yale University Press. 1973.
7. **INABA, Y., Y. TANAKA, K. SATO, H. ITO, T. OMORI and M. MATUMOTO.** Nomi virus, a virus isolated from an apparently new epizootic respiratory disease of cattle. Jap. J. Microbiol. 14: 246-248. 1970.
8. **JACOBS, J. W. and N. EDINGTON.** Isolation of respiratory syncytial virus from cattle in Britain. Vet. Rec. 88: 894. 1971.
9. **McKERCHER, D. G., J. K. SAITO, C. E. FRANTI, E. M. WADA and G. L. GRENSHAW.** Response of calves to parainfluenza-3 vaccines administrated nasally or parenterally. Am. J. vet. Res. 33: 721-730. 1972.
10. **MOTEANE, M., L. A. BABIUK and B. SCHIEFER.** Studies on the occurrence and significance of bovine respiratory syncytial virus in Saskatchewan. Can. J. comp. Med. 42: 246-248. 1978.
11. **PACCAUD, M. and C. JACQUIER.** A respiratory syncytial virus of bovine origin. Arch. ges. Virusforsch. 30: 327-342. 1970.
12. **PAVILANIS, V., J. H. JONCAS, R. SKOVORE, L. BERTHIAUME, M. F. BLANC, B. MARTINEAU et M. O. PODOSKI.** Microscopie électronique et diagnostic de laboratoire des maladies à virus. Union méd. Can. 100: 2195-2202. 1971.
13. **POTGIETER, L. N. D. and R. L. ALDRIDGE.** Use of indirect fluorescent antibody test in the detection of bovine respiratory syncytial virus antibodies in bovine serum. Am. J. vet. Res. 38: 1341-1343. 1977.
14. **ROSENQUIST, B. D.** Isolation of respiratory syncytial virus from calves with acute respiratory disease. J. infect. Dis. 130: 177-182. 1974.
15. **ROSSI, C. R. and G. K. KIESEL.** Serological evidence for the association of bovine respiratory syncytial virus with respiratory tract disease in Alabama cattle. Infection & Immunity 10: 293-298. 1974.
16. **SEVER, J. L.** Application of a microtechnique to viral serological investigations. J. Immunol. 88: 320-329. 1962.
17. **SMITH, M. H., M. L. FREY and R. E. DIERKS.** Isolation and characterization of a bovine respiratory syncytial virus. Vet. Rec. 94: 599. 1974.
18. **SMITH, M. H., M. L. FREY and R. E. DIERKS.** Isolation, characterization and pathogenicity studies of a bovine respiratory syncytial virus. Archs Virol. 47: 237-247. 1975.
19. **THOMAS, L. H., E. J. STOTT, A. P. COLLINS, N. J. JEBBETT and A. J. STARK.** Evaluation of respiratory disease in calves: comparison of disease response to different viruses. Res. vet. Sci. 23: 157-164. 1977.
20. **WELLEMANS, G., J. LEUNEN et E. LUCHSINGER.** Isolement d'un virus (220/69) sérologiquement semblable au virus respiratoire syncytial (RS) humain. Annls Méd. vét. 114: 89-93. 1970.
21. **WELLEMANS, G. et J. LEUNEN.** Le virus respiratoire syncytial et les troubles respiratoires des bovins. Annls Méd. vét 119: 359-369. 1975.