The characterization of a bovine rhinovirus

by

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A Thesis Submitted to the

Graduate Faculty in Partial Fulfillment of

The Requirements for the Degree of

MASTER OF SCIENCE

Department: Veterinary Microbiology and Preventive Medicine
Major: Veterinary Microbiology

Iowa State University
Ames, Iowa

1973

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I. INTRODUCTION

Bovine respiratory disease (BRD) remains the number one disease problem to the cattle industry in the United States. BRD is caused by a complex multiplicity of infectious agents including bacteria, mycoplasmas, chlamydia and viruses. Stress factors, nutrition, environment and many other factors play a role in the incidence of respiratory disease.

Many years of research have characterized the major causes of bovine respiratory disease. Potent vaccines are now available for the protection of animals against infectious bovine rhinotracheitis, parainfluenza-3, and bovine viral diarrhea.

An unending string of agents are being identified that are associated with BRD. Many of these agents, either alone or in combination with other agents, are capable of producing respiratory disease in cattle.

The purpose of this study was to characterize an isolant from a clinical case of BRD and to ascertain its role in BRD alone and with Mycoplasma agalactiae var bovis, a mycoplasma which commonly infects cattle.

II. LITERATURE REVIEW

A. History of Rhinoviruses

Kruse (44) first demonstrated that a common cold could be caused by nasal inoculation with a bacteria-free filtrate, indicating a viral etiology. The etiology of the common cold was studied further for many years before the isolation and identification of rhinoviruses. Recent work (77), however, indicated that most cases of the common cold were due to infection with these organisms.

In 1953 the DC strain (type 9) became the first rhinovirus to be propagated *in vitro* when it was passaged serially in explants of human embryonic lung in roller tubes (3). Tyrrell and Parsons (78) demonstrated that the viruses causing common cold would produce cytopathic effects in tissue culture of human embryonic kidney cells, if they were incubated in roller tubes at 33 C in a medium having low pH. The discovery of a semicontinuous strain of diploid human embryonic lung fibroblasts (26) initiated an explosive increase in the number of viruses isolated.

Many synonyms have been suggested for the viruses of the common cold: rhinoviruses (2); coryzaviruses (24); entero-like viruses (36); muriviruses and respiroviruses (52); and ERC (echo-rhino-coryza) group (42). Rhinovirus, derived from the special adaptation of these viruses to grow in the nose, is now the generally accepted name (4).

The World Health Organization initiated a Rhinovirus Collaborative Program in which all rhinoviruses were compared in cross neutralization tests with specific antisera. The first phase revealed 55 distinct serotypes and one subtype (38). This system was extended to 89 serotypes in 1971 (39). Recent isolations have been made in ciliated epithelial cells of human embryonic nasal epithelium maintained in organ culture. Isolants may be adapted to cell cultures after several passages in organ cultures, while others are unable to replicate in anything but ciliated epithelial cells (59,60). Preliminary data indicate that there are now 110 serotypes of human rhinovirus (35).

Rhinoviruses were isolated from horses by Plummer (61) and two distinct serotypes were isolated from cattle by Bogel and Bohm (9) and Reed et al. (66).

The International Committee on Nomenclature of Viruses (82) additionally included the foot-and-mouth disease virus as a rhinovirus. Newman et al. (56) proposed that rhinoviruses, foot-and-mouth disease virus and equine rhinovirus be placed into separate subgroups of the family Picornaviridae based on distinct differences of buoyant density in cesium chloride, stability at pH 3 to 7 and the base composition of the viral RNA. Foot-and-mouth disease virus, an acid labile picornavirus primarily of cattle was considered a rhinovirus, with the primary criterion being acid lability (62). However, it caused a disease quite different from that produced by other

rhinoviruses. Equine rhinovirus differed from other rhinoviruses in that it would multiply in a wide variety of cells (11), was not inhibited by (S,S)-1,2 bis(5-methoxy-2-benzimazolyl)-1-2-ethanediol (70) and, rather than being host specific, had been shown to infect human beings (61) and vervet monkeys (47).

Human rhinovirus research has been adequately reviewed (1,25,35,73,74,76) and is not detailed herein except when relevant to the subject.

B. Classification of Rhinoviruses

The name picornaviruses was proposed in 1963 (51) to include all small (pico) viruses that contain infectious ribonucleic acid (RNA). The viruses of the family Picornaviridae were found to conform to the cryptogram R/1 : 1-2.5/30: S/S : V/O (82). They were particles 20 to 40 nm in diameter of cubic symmetry containing single stranded RNA of molecular weight 2.5 X 10^6 daltons and were non-enveloped (naked). Viral synthesis and maturation occurred in the cytoplasm with the occasional formation of crystalline arrays of particles (82).

The family Picornaviridae was subdivided into genera Enterovirus (enterovirus, poliovirus, coxsackievirus and echovirus), Rhinovirus (human, equine, bovine and foot-and-mouth disease virus), and Calicivirus (vesicular exanthema and feline picornavirus) (82). Based on distinct differences of

buoyant density in cesium chloride, stability at pH 3 to 7 and the base composition of the viral RNA it was proposed that the family Picornaviridae more correctly should be divided into at least six subgroups: enteroviruses; rhinoviruses; caliciviruses; cardioviruses; foot-and-mouth disease virus; and equine rhinovirus (56).

Rhinoviruses are the largest genus of the family Picornaviridae and conform to the cryptogram R/1 : 2.6-2.8/30 : S/S : V/O (82). Members of the genus Rhinovirus are particles 20 to 30 nm in diameter with cubic symmetry and are non-enveloped (naked). They have single stranded RNA with a molecular weight of 2.6 to 2.8 X 10^6 daltons, which is about 30 percent of the total particle weight, with a base composition of G 20-24: A 26-34 : C 20-28 : U 22-26. They are labile at pH 3, ether and chloroform stable and some are stabilized against thermal inactivation by $MgCl_2$. They have a buoyant density of 1.38 to 1.43 g/cm^3 and a sedimentation coefficient of 140 to 150 S. They replicate in the cytoplasm of cells of the respiratory tract (82).

C. Bovine Rhinoviruses

Bogel and Bohm (9) first isolated a bovine rhinovirus in Germany (strain Sd-1). Subsequently, Wizigmann and Schiefer (83) isolated strain 181/V in Germany. In England Ide and Darbyshire (29) isolated RS 3x and Reed et al. (66) isolated EC11. Mohanty and Lillie (54) first isolated bovine rhino-

virus, strain C-07, in the United States (Maryland) and Rosenquist (68) made subsequent isolations in Missouri.

Most bovine rhinoviruses were serologically related, in contrast to the large number of human serotypes. Mohanty and Lillie (54) found that C-07 was related but not identical to Sd-1 and 181/V. Antiserum prepared against C-07 neutralized Sd-1 and 181/V at 8-and 16-fold lower titers, respectively, than the homologous strain. Ide and Darbyshire (34) concluded that strains Sd-1, 181/V, C-07 and RS 3x were antigenically similar. It was found that RS 3x was more closely related to Sd-1 strain than to C-07 strain. Betts et al. (5) found that serum from a calf infected with EC11 strain neutralized strain EC11, but not strain Sd-1. It appeared that strains Sd-1, 181/V, C-07 and RS 3x were all antigenically related, and were antigenically distinct from strain EC11 (34).

No serological relationship has been demonstrated between the bovine rhinoviruses and human rhinoviruses (8).

1. Isolation and growth in tissue and organ cultures

Human rhinoviruses have been grouped according to their ability in early passage to produce cytopathic effect only on human cell cultures (H strains), or on both monkey and human cells (M strains), the latter was comprised of relatively few serotypes (76). Another term, O strains, was used recently for viruses which were cultivated in organ cultures of human respiratory epithelium. While some strains replicated only in

organ cultures, others could be adapted to replicate in human embryo fibroblasts, and were then referred to as O-H strains (76).

The bovine rhinoviruses were shown to possess a very narrow host range comparable to the human rhinoviruses of the H type. Bovine rhinoviruses were found to replicate preferentially in calf kidney cells (8). Bovine rhinovirus isolations were made in stationary cultures of bovine kidney cells (9,54, 68,83). Ide and Darbyshire (29) utilized rolled cultures of bovine kidney cells in the isolation of RS 3x. Reed et al. (66) isolated strain EC11 in calf tracheal organ cultures. Only after eight passages in organ culture could the virus be adapted to calf kidney cells.

There is limited information available that indicates that the bovine rhinoviruses are tissue specific as well as species specific with regard to the cells in which they will replicate. Cytopathic effect was reported (30) in primary calf testis cultures, which on initial passage was transient. Virus replication occurred in primary calf thyroid cells without cytopathology until the third passage, when the cytopathic effect became identical to that found in calf kidney cells (30). Using Madin Darby bovine kidney cells (MDBK) a cytopathic effect similar to that found in primary bovine kidney cells was observed, but a very low viral titer was obtained

(66). MDBK cells were used in plaque assay of strains Sd-1, 181/V, C-07 and RS 3x (34).

Bogel (8) determined that a minimum of three passages at five day intervals should be made before a sample is considered negative for bovine rhinoviruses. On initial isolation only a few rounded cells were seen in focal areas (68). These cells were subsequently released from the monolayer (8). After several passages CPE could be detected as early as two days (68).

Bovine rhinoviruses replicated in organ cultures of trachea and turbinate explants with a resultant patchy cell damage characterized by loss of cilia, cellular disorientation and epithelial cell desquamation (30).

No replication of bovine rhinovirus was detected in bovine testicle (68) or amniotic and lymph node cells (8). Cells derived from heterologous species, including porcine kidney, guinea pig kidney, mouse kidney, chicken fibroblasts (8); HeLa, Vero, hampster kidney (BHK), rat embryo kidney, or L cells (30); and, Hep-2, primary human embryo kidney, primary monkey kidney and diploid semicontinuous human embryo lung cells (66), did not exhibit any cytopathic effect or demonstrable replication of bovine rhinovirus. Additionally, clinical signs were not evidenced in mice inoculated intraperitoneally, intracerebrally and subcutaneously. Guinea pigs showed no clinical signs after inoculation, and the virus

could not be reisolated after inoculation by several different routes in embryonating chicken eggs (8). Chickens and rabbits did not show clinical signs following intravenous inoculations (54).

One of the first successful demonstrations of the presence of rhinoviruses in vitro was by the interference technique (27). The utilization of the interference of non-cytopathogenic rhinoviruses against echovirus type II in human embryo kidney cells allowed the isolation and early characterization of rhinoviruses which would not cause detectable cytopathic effect until after at least 12 passages in culture (57).

Rhinoviruses were not isolated with ease until the inoculated cultures were rolled during incubation at 33 C and maintained in medium containing less than 0.09% sodium bicarbonate buffer (78). No disadvantage with the stationary incubation of bovine rhinoviruses was noted by Bogel (8). However, the extent of cytopathic effect was increased further by rolling the cultures according to Ide and Darbyshire (30). Cytopathic effect appeared earlier in rolled cultures, was more advanced at three days after inoculation and, in 50 percent of inoculated cultures was more advanced at six days. Cultures at 37 C had the least advanced cytopathic effect throughout the period of incubation (68).

The cytopathic effect produced by bovine rhinoviruses appeared more rapidly and was more progressive in cultures incubated at 33 C than in those incubated at 37 C. The virus titers obtained at 33 C were higher than when incubated at 37 C (9,30,54,68,83). The effect was probably due to a more rapid thermoinactivation of the viruses at 37 C (30). Inactivation occurred at the lowest temperature that inactivated the least stable component, either coat protein or nucleoprotein. $MgCl_2$ stabilized strain C-07 against thermoinactivation but strains Sd-1, 181/V (54) and RS 3x (31) were not stabilized.

Bogel (8) observed that the maintenance medium for culturing rhinoviruses must have a pH of 6.8 to 7.3. The utilization of a low bicarbonate buffer concentration (less than 0.09%) had long been recognized as a requirement to produce cytopathic effect in human embryonic kidney and monkey kidney cell cultures (78). In human diploid and HeLa cell cultures the incubation temperature and bicarbonate concentrations were less critical (24). The most sensitive cells for primary isolation of rhinoviruses (semicontinuous human lung and kidney fibroblasts) had a less critical medium pH requirement (37). The final titers with high and low bicarbonate concentrations (30) were found to be equal although maximal titers were attained more rapidly and the CPE was more pronounced with low bicarbonate concentrations. The CPE with high bicarbonate level often was obscured by regrowth of the monolayer.

Cultures at low bicarbonate concentration did not regrow, but were more acid and had non-specific cell degeneration (68).

Under optimal conditions for the replication of rhinoviruses, cytopathic effect could be observed in a monolayer of cells grown on the surface of a tube or flask. Under such conditions CPE appeared from 18 hours to two weeks after inoculation depending on the concentration and type of rhinovirus. When high concentrations were used CPE was diffuse but in low concentrations small foci appeared, which enlarged until they coalesced with other foci. These foci or microplaques were apparently each initiated by a single infectious viral particle (58).

The initial CPE evident in infected cells was an irregularity in outline, a rounded appearance and increased refractiveness (68). Reed et al. (66) described small foci consisting of six to ten rounded, refractile cells on an intact monolayer. The cells either lyse or detach from the surface as the CPE progresses in a slow manner that finally involves from 50 to 75 percent of the monolayer (68). Gaps appeared in the monolayer until a stage was reached where a lacy appearance was produced by narrow strands and islets of intact cells outlined by rounded and distorted cells (66).

Cellular changes were characterized by a retraction of cytoplasmic boundaries, often with an exhibition of intensely eosinophilic cytoplasmic masses that displaced the nucleus (which became pycnotic) and finally a complete cell shrinkage

(30). Infected cells stained with acridine orange exhibited flame red cytoplasmic masses that corresponded in location and morphology to granular cytoplasmic areas seen in cells stained with hematoxylin and eosin (30,31).

Electron micrographs of cells infected with human rhinovirus type 2 exhibited the appearance of large clusters of ribosomes (polyribosomes), the development of multiple cytoplasmic vesicles containing membrane bound bodies and the formation of dense granular intracytoplasmic material. These bodies proliferated until the nucleus was displaced. Progeny virions were recognized associated with parallel rows of fibrils and were also exhibited as crystals of closely packed viral particles (41).

Bovine rhinovirus RS 3x replicated in both trachea and turbinate organ cultures (30). Strain EC11 was isolated using trachea organ cultures and could not be adapted to bovine kidney cells until after the eighth organ culture passage (66). The ciliary activity of infected epithelial cells diminished and ultimately ceased (66). Histologically the infected organ cultures became non-ciliated and low columnar, cuboidal or squamous in appearance (30). The cellular damage was patchy and characterized by marked destruction and shedding of ciliated cells (65,66) with a disruption of normal cellular orientation (30). Peak titers of virus coincided with the cessation of ciliary activity (30).

Plaque assay for rhinoviruses was first described by Parsons and Tyrrell (58) who counted microplaques under a fluid overlay. Macroplaques were demonstrated under an agar overlay by Porterfield (63). Fiala (16) plaqued all of the first 55 serotypes of human rhinovirus utilizing 30 mM $MgCl_2$ and 30 µg/ml of DEAE-dextran in an overlay of agarose. Ide and Darbyshire (34) plaqued bovine rhinovirus strains RS 3x, 181/V, C-07 and Sd-1 under 1.2% Noble agar. Rosenquist (68) was unable to produce plaques in bovine embryonic kidney cells with his isolants.

The rate of rhinovirus RNA synthesis was increased when a PPLO medium was utilized for culturing rhinovirus infected cells. The synthesis of rhinovirus RNA has also been found to be greater in cell cultures infected with Mycoplasma pneumoniae (18,50). Additionally, Reed (64) showed that dual infection of tracheal organ cultures with bovine rhinovirus strain Sd-1 and Mycoplasma hyorhinis resulted in M. hyorhinis replicating more rapidly and to a higher titer than in control cultures.

2. Physical and chemical properties of bovine rhinoviruses

Stott and Killington (73) observed that relatively little was known about the replication and other properties of bovine rhinoviruses.

Bogel (8) calculated the size of bovine rhinovirus to be 25 nm according to the sedimentation constant. Mohanty and

Lillie (54) determined the size to be 25 to 27 nm according to electron micrographs and Ide and Darbyshire (31) estimated a size of 24 nm based on filtration.

The nucleic acid was determined to be ribonucleic acid (RNA) by several investigators. Rosenquist (68) determined the nucleic acid type in the direct method (23) using 5-bromodeoxyuridine (BUDR) and thymidine and in the indirect method (24) using BUDR alone. The RNA content of bovine rhinoviruses was determined additionally by indirect methods using 5-iododesoxyuridine (IUDR) (8,31), 5-bromodeoxyuridine (BUDR) (66) and 5-fluorodeoxyuridine (FUDR) (54).

Bogel (8) determined the sedimentation constant of bovine rhinovirus to be ca. 145 S. Ide and Darbyshire (31) found that the buoyant density in cesium chloride was ca. 1.40 gm/ml.

The resistance of bovine rhinoviruses to lipid solvents indicated that lipids were not an essential structural element (8). Bogel (8) found bovine rhinovirus to be resistant to chloroform and sodium-dodecyl-sulfate (SDS). Ide and Darbyshire (31) observed an increase in titer with chloroform treatment. Reed et al. (66) found bovine rhinovirus resistant to ether treatment. Sodium desoxycholate did not reduce infectivity (31). Ide and Darbyshire (31) found a marked reduction of infectivity when bovine rhinovirus was exposed to sodium-dodecyl-sulfate (SDS) for one hour. Viruses inacti-

vated by SDS usually, but not always, were those that contained lipids (81).

Bogel (8) found that strain Sd-1 was trypsin resistant. Ide and Darbyshire (31) found strain RS 3x to be trypsin sensitive and suggested that bovine rhinoviruses were variable in sensitivity to this enzyme.

Bogel (8) determined that the acid stability of isolants would differentiate between enteroviruses and rhinoviruses. Ide and Darbyshire (31) showed that rhinoviruses were stable at pH 6.0 to 8.0 but rapidly lost infectivity outside this range. Rosenquist (68) found incubation at 4 C to be necessary to preclude thermal inactivation while determining acid lability.

Rosenquist (68) found bovine rhinovirus isolants to be very heat sensitive. Ide and Darbyshire (31) demonstrated a significant difference between inactivation at 33 C and 37 C. The titer of rhinovirus at 37 C dropped significantly within six hours (8). Ide and Darbyshire (31) reported loss of approximately $10^{1.0}$ $TCID_{50}$ infectivity after 40 days storage at 4 C, $10^{0.5}$ $TCID_{50}$ at -20 C for one year and no loss of titer at -70 C for one year.

Mohanty and Lillie (54) found that strain C-07 was stabilized against thermal inactivation at 50 C by molar $MgCl_2$, whereas strains Sd-1, 181/V (31,54) were not stabilized.

3. Serology of bovine rhinoviruses

Hyperimmune sera against bovine rhinoviruses have been produced in guinea pigs, rabbits, calves (34) and chickens (54). Mohanty and Lillie (54) reported repeated failures to produce hyperimmune sera in guinea pigs and rabbits.

Rosenquist (68) reported at least an eight-fold increase in neutralizing titer in paired sera from three naturally infected animals. Bogel (8) found that active immunity produced low to moderate titers (1:2 to 1:32). Infection with bovine rhinovirus was shown to occur in the presence of relative high serum titers.

Neutralization tests with calf kidney cells in tubes (8, 66,68) and plaque reduction test (34) have been utilized for serological tests. Bogel (8) concluded that most other tests would be difficult to apply to bovine rhinovirus because of the relative low yield of virus in tissue culture.

Ide and Darbyshire (33) found that neutralizing antibody to bovine rhinovirus was passively transferred in colostrum. The titer initially dropped and then an average ten-fold increase occurred as the animal was naturally exposed to the virus.

Serological investigations have indicated that a high proportion of cattle possess neutralizing antibodies to bovine rhinovirus. Bogel (8) reported 67 percent incidence, Mohanty

and Lillie (54) 95 percent incidence and Ide and Darbyshire (33) 92 percent incidence of tested sera positive for rhinovirus antibodies.

4. Pathogenesis and pathology of bovine rhinovirus infection

Bogel (8) found bovine rhinovirus infection to be widespread in cattle but believed that these viruses were rarely associated with respiratory syndromes. Mohanty and Lillie (54), Ide and Darbyshire (29) and Reed et al. (66) isolated bovine rhinoviruses from apparently normal cattle. Rosenquist (68) recovered four isolants from cattle that had clinical signs of respiratory disease. Significant increases in titer to the homologous isolants plus the failure to detect other pathogens serologically or by isolation techniques suggested rhinoviruses as the causal agent in the infection.

Several workers have failed to produce symptoms of respiratory disease in calves inoculated with bovine rhinovirus (9,32,49,83).

Betts et al. (5) observed clinical signs consisting of a diphasic temperature increase of 1 to 2 C with peaks at 36 to 48 hours and four days post inoculation. An increase in the respiration rate accompanied the rise in temperature and a serous nasal discharge was observed from the second and third day following inoculation. Mohanty et al. (55) additionally observed coughing, hyperpnea, dyspnea, anorexia and central nervous system depression. The serous nasal discharge became

mucopurulent and on auscultation there were inspiratory and expiratory rales. Mohanty et al. (55) and Betts et al. (5) observed that the animals were not obviously ill and the infection might not be detected clinically.

Ide and Darbyshire (32) found no macroscopic lesions of nasal mucosa, tracheal mucosa, tonsils or bronchial and mediastinal lymph nodes. Betts et al. (5) observed small (less than 2 cm), dark red areas of collapse in the lungs. Mohanty et al. (55) observed macroscopic lesions typical of respiratory illness. The diaphragmatic and cardiac lobes of the lungs had areas of consolidation. Additionally, the turbinates and tracheal mucosa were congested.

Betts et al. (5), on histological examination, saw focal necrosis of turbinate epithelium. Adjacent cells were swollen and became non-ciliated. Ide and Darbyshire (32) found the normal pseudostratified columnar, ciliated cells replaced by low cuboidal, non-ciliated cells and cells characteristic of focal squamous metaplasia. Affected cells (5) demonstrated cytoplasmic vacuolation and degenerative changes of the nucleus. Serous glands showed degenerative changes characterized by pycnotic nuclei, perinuclear clearing of the cytoplasm and excessive granularity of the cytoplasm. Leukocytes were observed in the epithelial layer (5) and sub-epithelial connective tissue (32).

Betts et al. (5) reported necrotic foci involving only three to four epithelial cells in the trachea.

Mohanty et al. (55) found histological changes indicating pneumonia of varying severity. Betts et al. (5) found small areas of lobular collapse, microatelectasis and interstitial pneumonia in gnotobiotic calves.

Mohanty et al. (55) described alveoli filled with eosinophilic exudate and containing cellular debris. The alveolar walls were thickened by mononuclear infiltration. Large peribronchial lymphoid aggregates and masses of monocytes were found in areas with active proliferation manifested by pseudoepithelialization. There was a complete loss of alveolar structure. Edema and hemorrhage was observed in some alveoli. Large areas of atelectasis and emphysema were also observed.

III. METHODS AND MATERIALS

A. Source of Agents

1. Bovine rhinoviruses

The bovine rhinovirus FS1-43 used in this study was isolated via nasal swabs from one of six animals in a herd exhibiting signs of bovine respiratory disease.

The bovine rhinovirus VC96 was provided by Dr. B. D. Rosenquist, University of Missouri, Columbia, Missouri.

The bovine rhinovirus C-07 was provided by Drs. S. B. Mohanty and M. G. Lillie, University of Maryland, College Park, Maryland.

2. Bovine mycoplasma

Mycoplasma agalactiae var bovis (MAB) strains were provided by Dr. M. L. Frey, Veterinary Medical Research Institute, Iowa State University, Ames, Iowa.

B. Cell Culture Methods and Materials

1. Cell and organ culture materials

Various bovine cell types were utilized during this study to determine their ability to support the replication of bovine rhinovirus. Low passage bovine synovial, aorta, lung and tracheal cells obtained from a newborn calf; primary testicle cells obtained from a local slaughter house; low passage (31+) and high passage (101+) Madin Darby bovine kidney cells (MDBK); bovine embryonic kidney cell line (GBK) (71); bovine

turbinate cells (EBTu); and an epithelial cell which was isolated in pure culture from the fibroblastic EBtu's.

Bovine tracheal explants obtained from a newborn calf were utilized.

2. Cell culture methods

All cell culture procedures were conducted under a positive pressure plexiglass hood.

Cell cultures utilized in this study were routinely grown and maintained in Dulbecco's modification of Eagle's minimum essential medium (MEM) in 250 ml plastic cell culture flasks. The growth medium was supplemented with 10% heat inactivated (56 C for one hour) fetal calf serum (FCS) and the maintenance medium was supplemented with 2% FCS. The medium was buffered with 0.075% sodium bicarbonate and a mixture of organic buffers: N,N'-bis(2-hydroxethyl)2-aminoethanesulfonic acid (BES), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and N-2-hydroxyethylpiperazinepropane sulfonic acid (HEPPS) in final concentrations of 10, 15 and 10 mM, respectively (14). Cultures were incubated routinely at 37 C in 5% CO_2 atmosphere. Occasionally cells were incubated without increased CO_2 in a medium consisting of MEM buffered with N-tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) in sealed containers.

Cells were subcultured as needed for viral work or at intervals to maintain cell viability. To subculture cells,

they were first washed three times with 2.5 ml Rinaldini enzyme solution (R-saline), a calcium- and magnesium-free Tyrodes-like solution (67). The cell surface was flooded with 2 ml RTV (R-saline, 0.1% trypsin and 0.02% versene) until the cells were rounded-up. The RTV was removed and the flask was incubated at 37 C. After about two minutes the monolayer was dispersed with a firm rap of the flask on the table top. The cells were suspended in the desired quantity of medium and dispensed to recipient containers.

Routinely cells were dispensed into plastic tissue culture tubes placed in a horizontal position in stationary racks for incubation. On occasions tubes were placed into roller drums for incubation.

Antibiotics were not utilized unless isolation attempts were being made from nasal swabs. Crystalline penicillin G (100 IU/ml), dihydrostreptomycin (100 µg/ml) and amphotericin B (12.5 µg/ml) were utilized for control of contamination, but were omitted in subcultures.

Whenever it was feasible an attempt was made to inoculate cells with virus immediately after passage of the cells so that the cells were still in suspension (75). For certain procedures a monolayer was established prior to inoculation with virus. When monolayers were to be infected, the growth medium was removed and the cell sheet was washed with R-saline. The appropriate viral dilutions were added and the

tubes were labelled. After adsorption for one hour at 37 C the inoculum was poured off and one ml of maintenance medium was added. Cells were examined daily for cytopathic effect with an inverted microscope.

3. Storage of cell stocks

Stock supplies of cells were maintained in liquid nitrogen for storage. Cells were prepared for storage by suspending them in medium containing 20% FCS and 10% dimethyl sulfoxide (DMSO), by volume, after which they were sealed in glass containers. The cells were brought slowly to -70 C and then immersed in liquid nitrogen until needed.

4. Tracheal explant culturing methods

Tracheal explants were placed in wells of Linbro plates on areas that had been scratched to facilitate adherence to the plastic. The explant was flooded with MEM containing 20% FCS. Inoculation was accomplished by dropping the inoculum directly onto the explant, and then replacing the medium. Explants were observed daily for ciliary activity with an inverted microscope.

5. Plaque assay and purification

Plaques were produced using a modification of the method of Fiala (16). Dense monolayers of cells were established in chambers of Linbro plates. The cells were washed three times with R-saline and viral dilutions were added to the appropriate chambers. Sufficient MEM was added to produce a meniscus

that would facilitate an even distribution of virus on the monolayer. The virus was incubated for a minimum of three hours at 37 C to allow adequate adsorption. The fluid was removed and the cell surface was rinsed with R-saline. An overlay of 1% agarose mixed with an equal volume of 2X MEM with 4% FCS and antibiotic was applied at a temperature of 43 C. The overlay was allowed to solidify and the plates were then incubated at 37 C for 72 hours. The cells were fixed by flooding the overlay with 4% formalin for ten minutes. The agarose overlay was picked free and removed. The cells were stained by flooding with 1% crystal violet for three minutes. After drying the plaques could be observed with a background light or with an inverted microscope.

For plaque purification, isolated microplaques were marked while viewing with an inverted microscope. The plaques were then picked by plunging a Pasteur pipette into the center of the microplaque and applying suction until the agarose plug was lifted into the pipette. The material obtained was transferred to a culture tube. The procedure was repeated three times to plaque purify the virus.

The enhancement of plaque formation was determined for 30 mM/ml $MgCl_2$ (80), 30 µg/ml DEAE-dextran (17) and 0.5 mg/ml protamine sulfate (16). A combination of 30 mM $MgCl_2$ and 0.5 mg/ml protamine sulfate was used to determine its effect on plaque formation.

6. Mycoplasma cultivation

M-96 broth (21) supplemented with 15% swine serum (96Sw) was utilized for cultivation of the mycoplasmas used in calf inoculations, and for reisolation of mycoplasmas from experimentally infected calves.

C. Characterization of Isolant FS1-43

1. Determination of nucleic acid type

The nucleic acid type was determined with 5-bromodeoxyuridine (BUDR) according to the indirect method described by Hamparian et al. (24). Monolayers of GBK cells in tissue culture tubes were washed with R-saline and dilutions of virus were added with one ml MEM containing 10^{-4} BUDR. Parainfluenza-3 virus and infectious bovine rhinotracheitis virus, respectively, were used as RNA- and DNA-containing virus controls. The nucleic acid type was based on suppression of replication of DNA virus by BUDR.

2. Determination of chloroform sensitivity

The method of Feldman and Wang (15) was utilized to determine the sensitivity of the viral particle to lipid solvents. A mixture of .05 ml analytical reagent grade chloroform and one ml of tissue culture fluid containing virus (1:20) was shaken for ten minutes at room temperature. The mixture was centrifuged for five minutes at 500 rpm. The supernatant fluid was titrated for infectivity. Infectious

bovine rhinotracheitis and a reference strain bovine enterovirus, respectively, were used as enveloped and non-enveloped virus controls.

3. Determination of size

Filtration was conducted through sterile filters of pore size 0.22 µm (Millipore) and 0.05 µm (Gelman) using a modification of the method of Hsiuing (28). Ten ml of MEM with 10% FCS was first passed through the filter, this was followed by ten ml of tissue culture fluid containing virus. The viral content of the tissue culture fluid was titrated prior to and after filtration.

For negative staining a flask of infected cells was frozen and then agitated as they thawed. The tissue culture fluid was centrifuged at 5000 rpm for ten minutes. The cell precipitate was resuspended and the supernatant fluid was then centrifuged a second time at 35,000 rpm for one hour. Both the original precipitate and the final lower dense zone were prepared for examination using the method of Brenner and Horne (10). Twenty drops of distilled water, four drops of 4% phosphotunstic acid (PTA), one drop 0.1% bovine serum albumin (Cohn's fraction IV) and one drop of virus suspension were mixed in a depression dish. The mixture was applied to a carbon coated, collodian filmed 200 mesh grid with an all glass Vaponefrin type nebulizer. The grid was examined with a

Philips EM 200 electron microscope. The size was determined from the negative using a calibrated hand ocular.

4. Acid lability determination

Tissue culture fluid containing virus was diluted 1:10 in MEM and adjusted to pH 3.0 according to the method of Ketler et al. (42). The mixture was incubated at room temperature for 30 minutes after which the residual virus content was determined by titration. A reference strain bovine enterovirus was utilized as a control.

5. Thermostability

The method of Wallis and Melnick (80) was utilized to determine any stabilization by 1 M magnesium chloride. A mixture of 2 M magnesium chloride ($MgCl_2 \cdot 6H_2O$) was prepared in MEM to a final pH of 7.2. The virus was mixed with equal volumes of 2 M magnesium chloride or with equal volumes of MEM as a control. Portions of the mixture were incubated at 50 and 56 C. At various intervals samples were cooled rapidly and titrated for infectivity.

Samples of virus stored at -70 C were periodically titrated for infectivity. The loss of infectivity of the virus in storage was determined.

6. Characterization of cytopathic effect of cell preparations

Monolayers of cells were established on microscope slides or coverslips for detailed examination of cellular pathology. Uninfected control cells and infected cells demonstrating

progressive stages of cytopathic change were stained with hematoxylin and eosin and May-Grünwald-Giemsa. Infected and uninfected preparations were examined with acridine orange stain (48). Unstained preparations were also examined.

Monolayers of cells were washed with R-saline and suspended with RTV at various intervals following infection. The cells were fixed with 2.5% glutaraldehyde (pH 7.4) for 25 minutes, washed twice with sodium cacodylate buffer (pH 7.4) and post-fixed with 1% osmium tetroxide for 30 minutes. The cells were then washed twice in sodium cacodylate buffer, dehydrated in ethyl alcohol and embedded in a Epoxy-resin mixture (45). Sections were made and stained with uranyl acetate and lead citrate (40). Sections were examined in a Hitachi HU-8 electron microscope.

D. Serological Test Procedures

1. Hemagglutination

Hemagglutination of bovine rhinovirus was performed according to the procedure reported by Stott and Killington (72). Erythrocytes were washed three times in the test buffer and resuspended to a concentration of 0.5% in a solution consisting of 50% glucose solution (4.5%), 50% veronal buffer (complement fixation-test buffer), and 0.05% gelatin. The final pH was adjusted to pH 5.0 with 0.2 M NaOAc-HOAc buffer, pH 6.0 with 0.05 M phosphate buffered saline, pH 7.0 with 0.05 M tris buffer or pH 8.0 with 0.05 M tris buffer. Erythrocytes

from human group-O, chicken, goose, rat, guinea pig, rabbit, bovine, sheep and equine were utilized after collecting one volume of blood in an equal volume of Alsever's solution. The virus was grown in GBK cells and centrifuged at 2000 rpm for five minutes to remove cell debris. The resultant supernatant fluid served as the hemagglutinin. Equal volumes of erythrocytes in suspension at the various pH's and hemaglutinin were mixed in micro-titer plates. The mixtures were incubated at 4 C, room temperature and 37 C.

2. Complement fixation test

Complement fixation was performed using the adaptation to micro-technique (79). A block titration with 0.25 ml of serial doubling dilutions of known positive serum and 0.25 ml of serial doubling dilutions of antigen were made in a micro-titer plate. Five $C'H_{50}$ units of complement in 0.25 ml volume was added and the plates were incubated at 4 C overnight. A volume of 0.25 ml of sheep erythrocytes (2.8% standardized cell suspension) sensitized to a standardized hemolysin solution was added. The mixture was incubated at 37 C for 30 minutes. Results were read after placing the micro-titer plate on a test reading mirror. Antigen, antisera and 2.5 $C'H_{50}$ controls were also prepared.

3. Serum neutralization test

A serum neutralization test in micro-titer plates according to the method of Gwaltney (22) and Kriel et al. (43) was

utilized. Serial doubling dilutions of serum (0.05 ml) were made in micro-titer plates. Twenty-five $TCID_{50}$ of virus (13) in 0.05 ml of MEM was added and mixed on a platform shaker. The plates were incubated for two hours at 37 C. Cells suspended in 0.05 ml MEM containing 10% FCS were added and the plates were incubated at 37 C. The plates were examined at two days for cytopathic effect with an inverted microscope. The serum titer (microplaque reduction titer) was expressed as the reciprocal of that dilution with two microplaques or less cytopathic change in the cell sheet (13).

4. Plaque reduction neutralization test

Serial two-fold dilutions of heat inactivated serum were mixed with equal volumes of virus suspension containing approximately 80 p.f.u. per 0.1 ml. After incubation for two hours at room temperature 0.1 ml was inoculated into paired wells in a Linbro plate containing a monolayer of GBK cells. Equal volumes of virus and diluent were inoculated as a control. All cultures were overlaid with agarose and incubated as described under plaque assay and purification. The serum titer was expressed as that dilution which effected a 50% reduction in plaque count.

5. Fluorescent antibody test

a. Hyperimmune serum production Rabbits were hyperimmunized with two ml virus suspension in MEM (chloroform treated) mixed with an equal volume of Freund's complete

adjuvant. The inoculum was injected in equal volumes at five sites, one intramuscular, two subcutaneous and two in the foot pads. One ml of virus suspension in MEM (chloroform treated) was injected into the lateral ear vein on days 21, 28, 29, 32, 33, 34 and 40. Serum was collected on day 47.

Chickens were hyperimmunized by inoculation of one ml virus suspension (chloroform treated) intravenously at six weekly intervals. Serum was collected at the end of seven weeks.

b. Preparation of conjugated antibody The gamma globulins in ten ml hyperimmune serum were precipitated with 6.1 ml saturated ammonium sulfate at 4 C. The precipitated globulin was washed two or three times with saturated ammonium sulfate. The precipitate was redissolved in a minimum volume of distilled water and dialyzed at 4 C against at least two changes of 0.9% NaCl - 0.05 M sodium carbonate (pH 9.0) for 24 to 36 hours. The protein content of the globulin was adjusted to 20 mg per ml. Conjugation of fluorescein isothiocyanate (FITC) with globulin was done by a modification of the method described by Marshall et al. (46). A solution of serum, 18 mg FITC per gram of protein and 1 M sodium carbonate (pH 9.0) in a volume of three ml per 18 mg FITC was incubated at 25 C for 90 minutes. The globulin-FITC solution was neutralized with 1 M NaH_2PO_4 and then dialyzed overnight against 0.01 M sodium phosphate buffer (pH 7.5) with three grams of 50

mesh Dowex added to pick up excess FITC. The conjugate was absorbed on a column of DEAE-cellulose, type 40 which was equilibrated with 0.01 M NaH_2PO_4 (pH 7.5). The column was washed with 0.01 M NaH_2PO_4-0.05 M NaCl (pH 7.5) to elute any low-labeled material. The desired globulin was eluted with 0.01 M NaH_2PO_4-0.4 M NaCl (pH 7.5).

c. Test procedure The direct method of fluorescent antibody technique as described by Coons and Kaplan (12) was used. Coverslips with a monolayer of infected and uninfected GBK cells were rinsed with fluorescent antibody buffer, fixed in cold acetone for 15 minutes and overlaid with labeled globulin for 30 minutes in a moist chamber at 37 C. The coverslips were rinsed in fluorescent antibody buffer and distilled water for ten minutes each. The coverslips were mounted in buffered glycerine. The slides were examined under a fluorescent microscope.

E. Serological Examinations

1. Serological survey

Acute and convalescent sera from animals clinically ill with symptoms of bovine respiratory disease were tested using the serum neutralization test described above. A seroconversion, indicative of recent infection with bovine rhinovirus strain FS1-43 was considered to be at least a two-fold increase in microplaque reduction titer.

2. Specificity of antibody for the virion

One ml of bovine serum containing antibody specific for bovine rhinovirus (strain FS1-43) was incubated overnight at room temperature with 0.1 ml of final viral preparation used in negative staining as described above. The mixture was centrifuged for one hour at 35,000 rpm. The dense lower zone was negatively stained and examined for antibody coated virions with a Philips EM 200 electron microscope as described above.

F. Animal Infectivity Studies

A yearling steer from a university-owned beef herd was examined for a serological titer and obtained for experimentation.

Calves from a university-owned beef herd and from two local dairy herds were obtained at birth and placed in individual stalls, isolated from other animals and personnel that had contact with other cattle. Serum (50 ml) from the first surviving calf was fed, within six hours of birth, to each subsequent calf as a source of protective antibodies. One ml of a solution containing 500,000 I.U. vitamin A, 75,000 I.U. vitamin D_2 and 50 I.U. vitamin E was administered intramuscularly. Three ml of a combination of procaine penicillin (200,000 I.U./ml) and dihydrostreptomycin sulfate (0.25 gm/ml) was administered twice daily for the first seven days. Powdered milk cultured with Lactobacillus acidophilus was fed for

the first three days and then replaced with a commercial milk replacer. The calves were weaned at one month of age and fed a commercial calf starter and alfalfa hay.

Calves were examined for a serological titer against bovine rhinovirus, and mycoplasma isolation was attempted using nasal swabs plated on 96Sw medium prior to experimentation.

1. Group one

A yearling steer (400) was inoculated with bovine rhinovirus strain FS1-43 for the purpose of passaging the virus in an animal to bolster the animal virulence of the virus, to develop isolation techniques and to serve as a preliminary investigation to determine the development of symptoms and pathological changes. The animal was inoculated with three ml of virus suspension intratracheally and intranasally with one ml of virus suspension. The virus suspension had a titer of $10^{4.0}$ $TCID_{50}$/ml. The steer was examined daily for clinical signs, respiration rate and rectal temperature.

Nasal swabs were collected daily, placed in two ml MEM with antibiotics for transport and centrifuged for five minutes at 2000 rpm. An inoculum of 0.1 ml was placed into tissue tubes with GBK cells suspended in 0.9 ml of MEM with antibiotics. Four tubes were incubated at 37 C in a stationary position, four at 34 C in a stationary position and four at 34 C in a rotor. Two tracheal explants were inoculated with

0.1 ml. A series of three passages were made before a sample was considered negative.

Serum was collected before inoculation and at 16 days post inoculation. The serum titer was determined utilizing the serum neutralization test.

At 30 days post inoculation the steer was slaughtered at the university meat lab and a post mortem examination was conducted for the detection of gross pathological lesions.

2. Group two

Two calves (410,411) were inoculated intratracheally with three ml virus suspension and intranasally with an aerosol of one ml virus suspension. The virus suspension contained rhinovirus at a titer of $10^{4.5}$ $TCID_{50}$/ml. Calf 409 was placed with the first two calves at 24 hours post inoculation as a contact control.

On day six post inoculation, calf 411 was isolated and inoculated intratracheally with one ml of supernatant fluid from a suspension of pneumonic lung. Organisms resembling Mycoplasma agalactiae var bovis, but no viruses, had been isolated from the lung suspension.

The calves were examined daily for respiration rate, rectal temperature and other clinical signs. Blood samples were collected daily and examined for total white blood cell count, differential white blood cell count, hematocrit, plasma

fibrinogen, total serum protein and protein ratio (total protein to plasma fibrinogen).

Nasal swabs were collected daily from day one post inoculation until eight days post inoculation. Swabs were placed in one ml MEM with antibiotics for transport, centrifuged at 2000 rpm for five minutes and 0.1 ml was inoculated into eight tubes. Suspended GBK cells were added in 0.9 ml MEM with antibiotics and incubated in a stationary position at 37 C. Three serial passages were made before a sample was considered negative.

At the end of the experiment the calves were electrocuted and a post mortem examination was conducted: calf 410 (rhinovirus) on day six post inoculation, calf 409 (contact control) five days after initial contact and calf 411 (rhinovirus and mycoplasma) six days after mycoplasma inoculation.

Calves were examined for gross pathological lesions and tissues were collected for microscopic examination.

Swabs were collected from hip, stifle and elbow joint fluid; lung; and cervical and mediastinal lymph nodes of calf 411, and isolation of mycoplasmas was attempted on 96 Sw solid medium. Lung tissue was collected from several sites, ground in a tissue grinder with M-96 broth, and mycoplasma isolation was attempted from 1/10, 1/50 and 1/500 dilutions plated onto M-96 solid medium. Serum was collected from calf 411 for the serum neutralization test.

3. Group three

Calf 412 was inoculated intranasally and intratracheally with four ml rhinovirus (10^4 $TCID_{50}$/ml) combined with one ml of M-96 medium containing 4 x 10^7 Mycoplasma agalactiae var bovis organisms.

At three days post inoculation calf 419 was placed in contact with calf 412 for 48 hours. At that time calf 419 was inoculated with the above inoculum. Additionally an inoculum prepared from fluid collected after administering five ml MEM into the nasal cavity of calf 412 was inoculated intranasally.

Calf 418 was inoculated intranasally and intratracheally with 4 x 10^7 Mycoplasma agalactiae var bovis organisms in four ml of M-96 medium. Calf 418 was isolated from all other animals.

Calves 421 and 422 were maintained as uninoculated control calves. They were isolated from all infected calves.

Samples were collected and processed as with group two. At the termination of the experiment the calves were electrocuted and a post mortem examination conducted as described above.

IV. RESULTS

A. Cell and Organ Culture

1. Growth studies in bovine cells

The replication of bovine rhinovirus FS1-43 in selected bovine cells is summarized in table 1. High passage MDBK

Table 1. Replication of bovine rhinovirus FS1-43 in bovine cells

Type of cell	Titer based on CPE[a,b]	Titer based on CPE in GBK cells after subculture[a,b]
Synovial strain	0	0
Aortic intima strain	0	0
Lung strain	1.75	3.5
Tracheal epithelium strain	0	0
Testicle strain	0	0
MDBK (low passage)	2.0 (stationary) 4.5 (rotated)	4.33 4.5
MDBK (high passage)	0	0
EBTu	1.0	2.67
EBTu-B	3.5	5.0
GBK	4.5	4.5

[a] Average of three experiments

[b] Titer stated as $\log_{10}TCID_{50}/ml$

cells and low passage strains derived from synovia, aortic intima, trachea and testicle did not develop CPE, within seven days, when inoculated with bovine rhinovirus strain FS1-43. GBK cells inoculated with 0.1 ml medium from these cells, after freezing and thawing two times, did not develop CPE within five days. After passage from each cell type to GBK cells without the development of CPE, the cells were determined to be refractory to bovine rhinovirus infection.

Lung and EBTu cells initially exhibited foci of CPE at 10^{-1} dilutions of virus. After eight serial passages in lung cells generalized CPE at $10^{-1.5}$ dilutions of virus was obtained. CPE in EBTu cells did not progress beyond foci of six to eight cells at 10^{-1} dilution of virus after eight serial passages. GBK cells inoculated with 0.1 ml of virus suspension harvested from the eighth serial passages in lung and EBTu cells developed CPE indicating titers of $10^{3.5}$ and $10^{2.67}$ $TCID_{50}$/ml, respectively.

Low passage MDBK, GBK and EBTu-B cells were found to support the replication of bovine rhinovirus strain FS1-43. Low passage MDBK cells at 24 hours had a CPE characterized by foci of rounded, translucent cells. At 48 hours culture tubes had a greatly increased amount of cellular debris in the medium, but no apparent CPE. Rotation of culture tubes prevented regrowth of the cell sheet and facilitated progression of recognizable CPE.

A comparison of replicate titrations in low passage MDBK, GBK and EBTu-B cells is summarized in table 2. To compare the

Table 2. A comparison of replicate titrations in selected cell types

Type of bovine cell[a]	Titer based on CPE[b]	Subculture titer based on CPE[b]		
		GBK	MDBK	EBTu-B
MDBK	5.0	5.0	-	ND
GBK	5.0	-	5.0	ND
EBTu-B	4.5	5.0	4.0	-

[a]Average of two experiments

[b]Titer stated as $\log_{10}TCID_{50}$/ml

relative ability of each cell type to support the replication of bovine rhinovirus, virus harvested from each cell type was titrated in GBK and MDBK cells. Titrations were not attempted in EBTu-B cells because of the inconsistent exhibition of CPE.

All cell types were consistently negative for *Mycoplasma spp.* on periodic culture attempts on 96Sw solid medium.

2. Comparison of different incubation conditions

Virus yield after incubation of replicate titrations of infected GBK cells at 33 C in stationary tubes, at 33 C in tubes rotated at one revolution per five minutes and at 37 C in stationary tubes were compared. An average of five experiments revealed very little difference in titers obtained (3.54, 3.40 and 3.60 $\log_{10}TCID_{50}$/ml respectively).

The time of initial CPE appearance, progression of CPE and final virus yield did not prove to be influenced by the temperature of incubation of infected cells. Rotation of infected MDBK cell cultures was required to prevent regrowth of the monolayer and the resultant inability to detect foci of CPE. The initial appearance of CPE and final virus yield was not affected by rotation of cultures or the temperature of incubation.

CPE was exhibited when EBTu-B cells were infected in a suspension of cells that would produce a sparse or incomplete monolayer. When a dense monolayer resulted CPE could not be detected beyond a 10^{-1} virus dilution. Rotation of cultures resulted in a dense monolayer of cells and no visible CPE. Temperature and rotation of cultures did not influence the time of appearance of CPE and final virus yield.

All titrations were determined at 72 hours after infection of cells. Further incubation did not alter the final titration based on CPE. Subcultures after 72 hours incubation indicated a lower titer of infectious viral particles harvested.

3. Growth studies in bovine tracheal explants

Ciliary activity of bovine tracheal explants was not affected by bovine rhinovirus isolant FS1-43 in each of three attempts. Medium harvested from bovine tracheal explants after 24 hours incubation produced CPE in GBK cells.

4. Plaque production

Bovine rhinovirus strain FS1-43 utilized in this study was successfully plaqued under an agarose overlay after a minimum of three hours incubation to allow the adsorption of the virus to the cells.

Seventy-two hours incubation at 37 C was found to be the optimum incubation period for plaque production. At that time distinct well delineated plaques were evidenced. With additional incubation time there was no increase in the clarity of the plaques. Even with seven days incubation some plaques did not become large enough to be identified as such without microscopic examination. After a long incubation time the plaques developed an indistinct border and adjacent plaques became fused making it difficult to accurately enumerate the plaques produced.

Prior to characterization of the virus and infectivity studies the rhinovirus isolant was purified by picking isolated plaques for a series of three procedures.

Efforts to distinguish and to isolate plaque variants were not successful. Microplaques and large plaques selected as possible variants produced plaques similar if not identical to those characterized as being normal plaques. Serial selections of apparent variants produced similar results.

Incubation at 33 C or 37 C did not affect the time of appearance of plaques, size of plaques or the final total plaque count.

Plaques could not be visualized with a neutral red overlay as utilized in most standard plaque techniques.

5. Enhancement of plaque formation

Plaques produced by rhinovirus after three to seven days incubation can be best described as pinpoint in size (figure 1). Large isolated plaques reached a maximum of 0.5 mm in diameter. Plaques were clear with an irregular border.

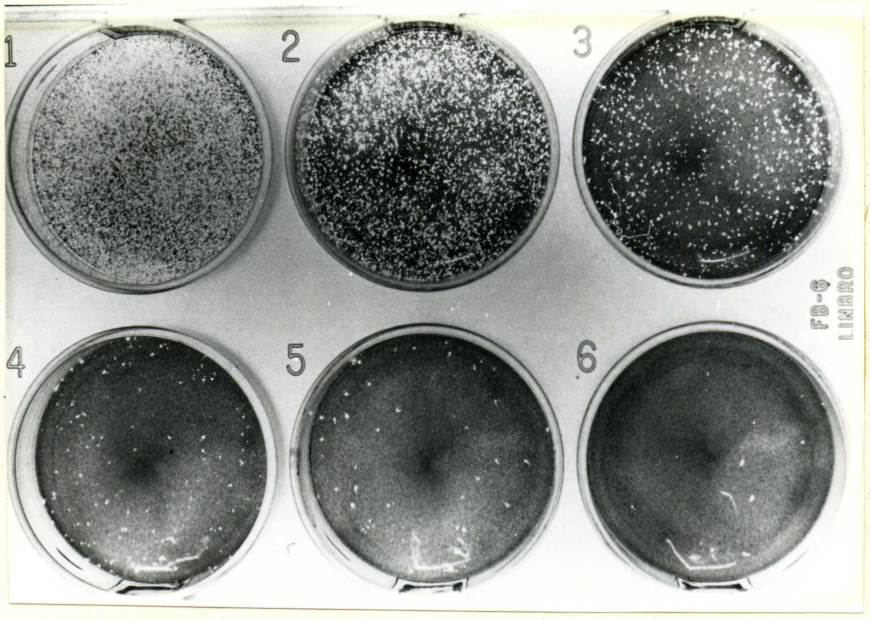
The addition of 30 µg/ml DEAE-dextran resulted in a loss of viability of the entire monolayer of cells. The monolayer was also disrupted when 15 µg/ml DEAE-dextran was added.

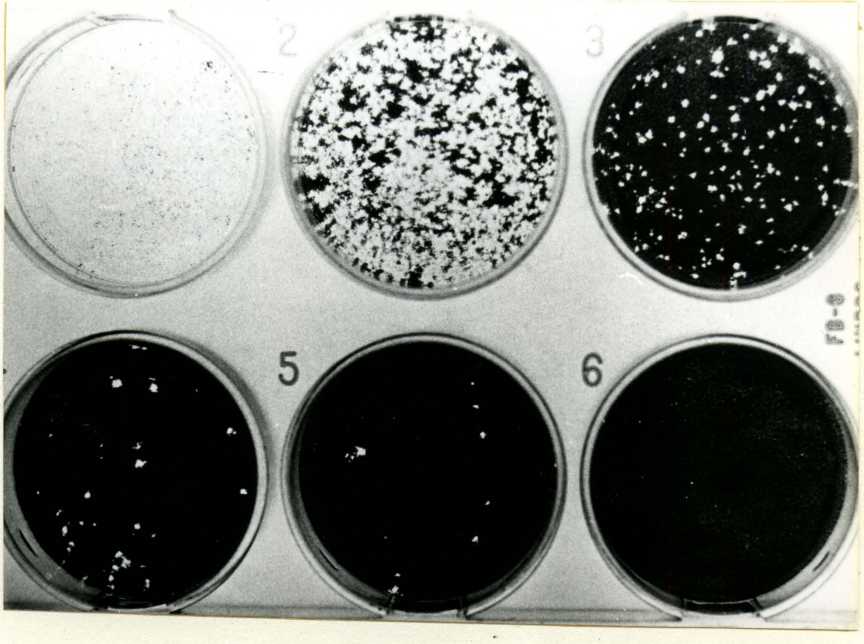
Protamine sulfate in a concentration of 0.5 mg/ml had an effect of causing an incomplete destruction of cells within a plaque and the development of an irregular outline giving the plaques a fuzzy appearance.

The addition of a concentration of 30 mM/ml $MgCl_2$ resulted in a two- to four-fold increase in plaque diameter (final diameter 1 to 2 mm). Plaques (figure 2) were slightly irregular in outline but had a distinct border with a complete destruction of cells within the plaque resulting in a distinct clear plaque.

Figure 1. Plaques of pinpoint size produced in GBK cells by rhinovirus strain FS1-43. Stained with 1% crystal violet. Actual size.

Figure 2. Plaques produced in GBK cells by rhinovirus strain FS1-43 after the addition of 30 mM/ml $MgCl_2$ to the agarose overlay. Stained with 1% crystal violet. Actual size.


1
2
3
4
5
6
FB-6
LINARO


2
3
5
6
FB-6

The addition of a combination of 30 mM/ml $MgCl_2$ and 0.5 mg/ml protamine sulfate produced plaques 0.5 to 2.0 mm in diameter that were fuzzy and with an irregular outline.

B. Characterization of Isolant FS1-43

1. Nucleic acid type

The nucleic acid type was indirectly determined to be RNA when the addition of 10^{-4} BUDR, a thymidine analog, to the medium did not result in a reduction in titer. Parainfluenza-3 virus (the control RNA virus) was not inhibited by BUDR but infectious bovine rhinotracheitis virus was markedly reduced in titer. Table 3 summarizes the results.

Table 3. Effect of BUDR on virus titer

Virus	Titer of control[a]	Titer after BUDR treatment[a]	Nucleic acid type
FS1-43[b]	3.77	3.77	RNA
Parainfluenza-3[c]	5.75	5.50	RNA
Infectious bovine rhinotracheitis[d]	7.08	4.67	DNA

[a]Titers stated as $\log_{10}TCID_{50}$/ml

[b]Average of five experiments

[c]Average of two experiments

[d]Average of two experiments

2. Chloroform sensitivity

Virus infectivity was not reduced by chloroform treatment and in four of five experiments it was increased. The titer of a bovine enterovirus reference strain (LCR-4) was not reduced but infectivity of infectious bovine rhinotracheitis virus could not be detected after exposure to the same concentration of chloroform for ten minutes. The results of chloroform sensitivity are summarized in table 4.

Table 4. Sensitivity to chloroform

Virus	Titer of control[a]	Titer after chloroform treatment[a]
FS1-43[b]	3.54	3.9
Bovine enterovirus LCR-4[c]	6.33	6.0
Infectious bovine rhinotracheitis[d]	8.33	<1.0

[a]Titers stated as $\log_{10}TCID_{50}/ml$

[b]Average of five experiments

[c]Average of two experiments

[d]Average of two experiments

3. Determination of size

The virus passed through 0.22 μm and 0.05 μm filters without a reduction in titer. A size of less than 32 nm is calculated with the application of Black's factor (6).

Negative stained viral particles (figure 3) were ca. 26 to 32 nm in diameter.

4. Acid sensitivity

After 30 minutes incubation in a medium of pH 3.0 no residual viral infectivity could be detected. A reference strain bovine enterovirus (LCR-4) similarly treated did not exhibit a reduction in titer. The results of acid sensitivity are summarized in table 5.

Table 5. Sensitivity to treatment at pH 3.0

Virus	Titer of control[a]	Titer after acid treatment[a]
FS1-43[b]	3.36	<1.0
Bovine enterovirus LCR-4[c]	6.0	6.0

[a]Titers stated as $\log_{10}TCID_{50}/ml$

[b]Average of five experiments

[c]Average of two experiments

5. Thermostability

Bovine rhinovirus inactivation curves (figure 4) show that 90 percent reduction of infectivity occurred in approximately 8.0 hours (37 C), 12 hours (34 C), 67 hours (22 C, room temperature) and 120 hours (4 C).

Figure 3. Bovine rhinovirus particle negatively stained with phosphotungstic acid. 598,500X.

Bovine rhinovirus stored at -70 C lost infectivity in a linear fashion as demonstrated in figure 5. The time for 90 percent reduction in infectivity was approximately 50 days as determined from the infectivity curve. Four separate stock virus preparations lost an average of 92 percent infectivity at an average of 78 days storage at -70 C as determined by titrations for infectivity.

Rhinovirus was not stabilized against thermoinactivation by 1 M $MgCl_2 \cdot 6H_2O$ at 50 C. Residual infectivity titers of $10^{1.5}$ and $10^{1.0}$ $TCID_{50}$/ml were obtained after 15 and 30 minutes, respectively, when a sample with an initial infectivity titer of $10^{3.5}$ $TCID_{50}$/ml was incubated with 1 M $MgCl_2 \cdot H_2O$ at 50 C. The controls incubated without added magnesium were negative for infectivity after 15 minutes incubation at 50 C.

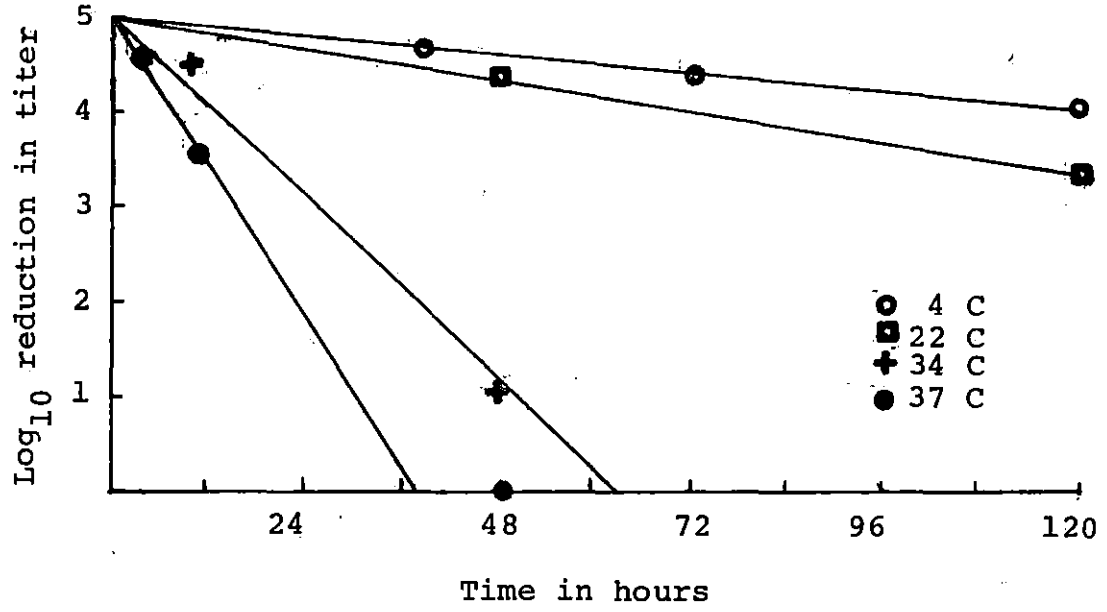
Samples with and without magnesium chloride were negative for residual infectivity after 15 minutes incubation at 56 C.

6. Characterization of cytopathic effect of cell preparations

In unstained cell preparations the cytopathic effect of bovine rhinovirus, figures 6 and 7, was first visualized by individual infected cells becoming rounded and shrinking. The cells become translucent and appear to be elevated above the cell sheet. Adjacent cells become affected and a small clump or focus develops. The foci progressively enlarge and coalesce with other foci. As the cells become rounded the

Figure 4. Thermoinactivation of strain FS1-43.

Figure 5. Stability of strain FS1-43 at -70 C.


Log10 reduction in titer
5
4
3
2
1
4 C
22 C
34 C
37 C
24
48
72
96
120
Time in hours

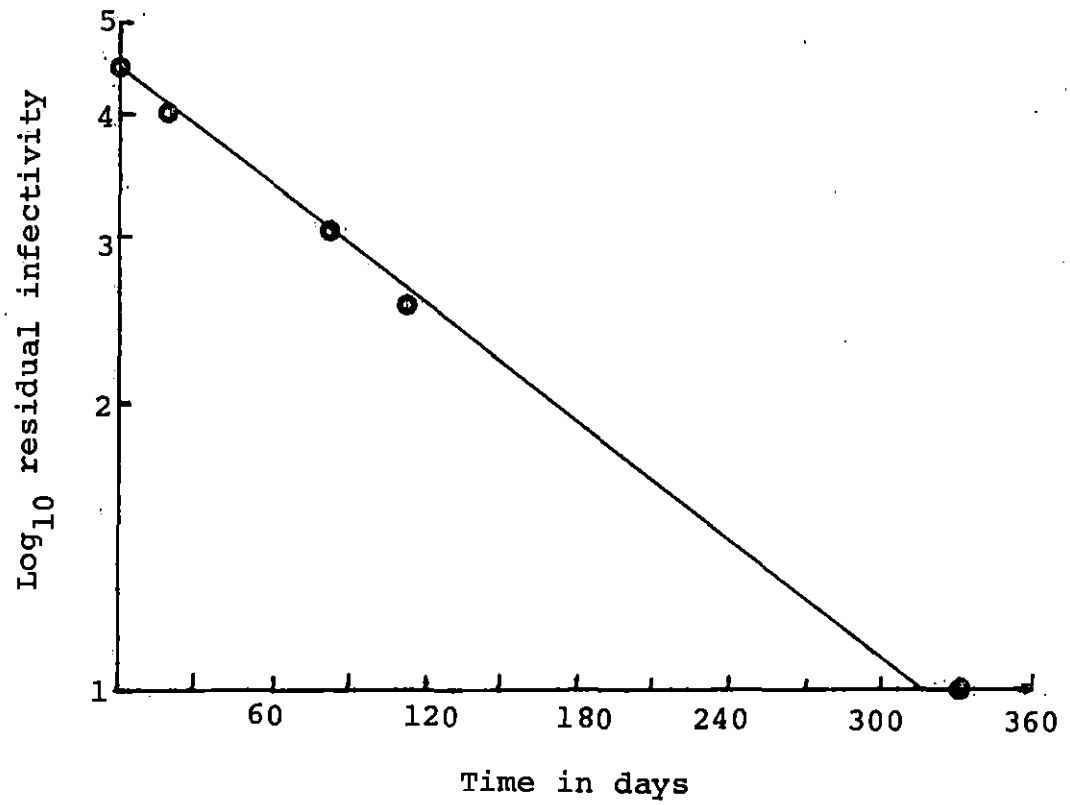
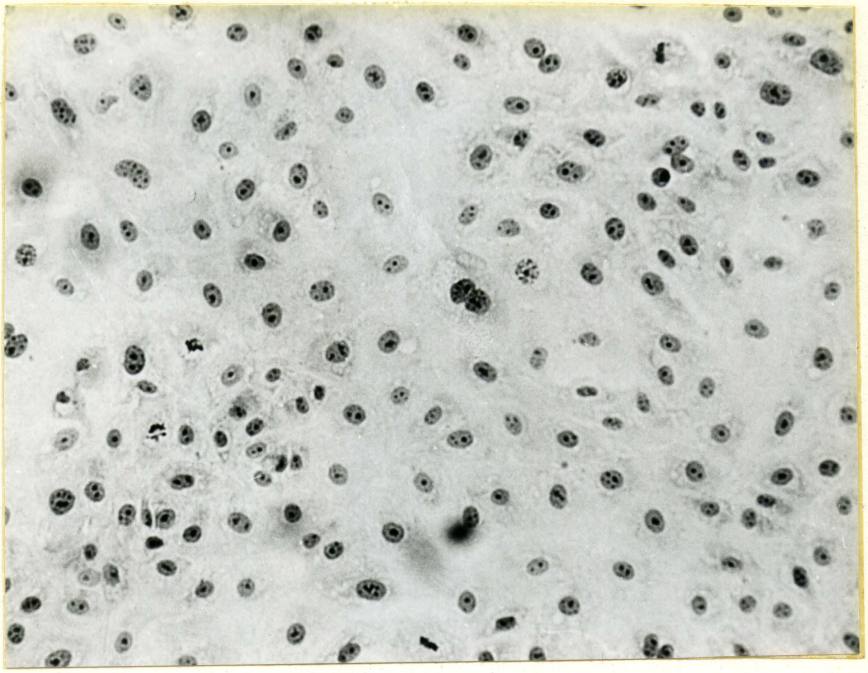
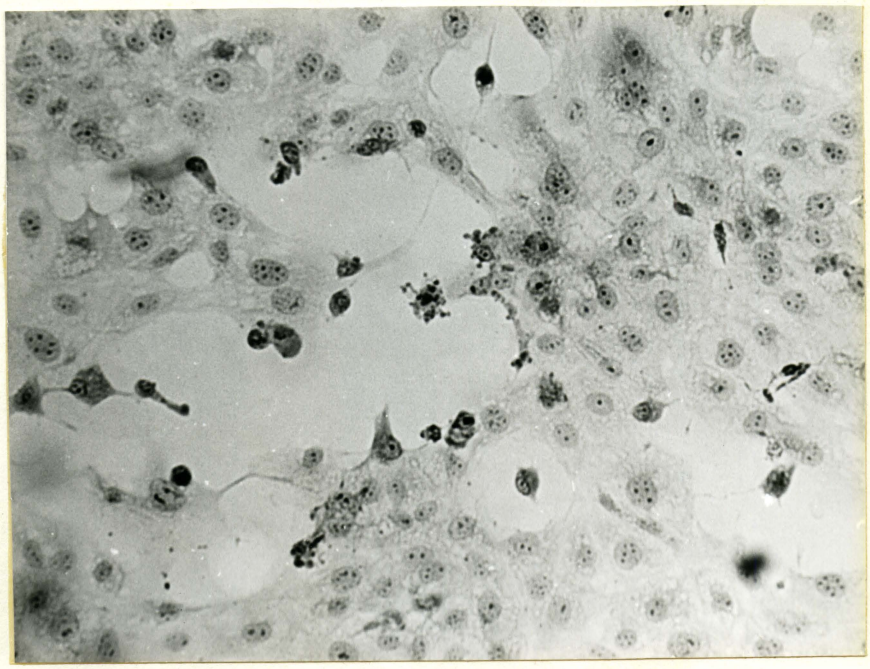

Log10 residual infectivity
5
4
3
2
1
60
120
180
240
300
360
Time in days

Figure 6. Uninfected GBK cells. Stained with hematoxylin and eosin. 430X.

Figure 7. GBK cells infected with bovine rhinovirus strain FS1-43 showing cytopathic change. Stained with hematoxylin and eosin. 430X.





cytoplasmic processes are retracted. The contracted cells are released from the cell sheet leaving a focus with a network of strands and islets of cells. The perimeter of the focus is fringed with contracting cells.

Stained preparations revealed a high degree of vacuolization of infected cells and frequently an eosinophilic granular cytoplasmic mass. The nucleus became displaced and pycnotic prior to shrinkage of the cell.

Acridine orange stained cells exhibited cytoplasmic areas that were more intensely flame-red than the remainder of the cytoplasm. These areas appeared to correspond with the eosinophilic masses observed in cells stained with hematoxylin and eosin. The nucleus was displaced in the cells exhibiting cytoplasmic masses.

Electron microscopic examination of uninfected cells revealed centrally located nuclei, numerous mitochondria throughout the cytoplasm and endoplasmic reticulum with some attached ribosomes. Ribosomes occurred in the cytoplasm, either singly or in groups of two or more particles (polyribosomes).

Infected cells developed clusters of ribosomes which formed dense masses. The endoplasmic reticulum became fully lined with ribosomes. The cytoplasm became highly vacuolated. Initially the vacuoles contained a membrane bound body with a cytoplasmic appearance. This body was not in evidence as the

vacuoles enlarged and proliferated but was replaced by scattered debris. The vacuolization progressed until the nucleus was displaced laterally. The nucleus, in addition to being displaced, showed lobulation and shrinkage.

Virions were not visualized in infected cells grown with or without the addition of 30 mM $MgCl_2$.

C. Serological Tests

1. Hemagglutination test

Bovine rhinovirus strain FS1-43 did not hemagglutinate human group-O, chicken, goose, rat, guinea pig, rabbit, bovine, sheep and equine erythrocytes at pH 5.0, 6.0, 7.0 or 8.0 at temperatures of 4 C, 22 C (room temperature) or 37 C.

2. Complement fixation test

Complement fixation activity was not detected with bovine rhinovirus strain FS1-43 and known positive serum.

3. Serum neutralization test

A serum neutralization test in micro-titer plates utilizing 25 $TCID_{50}$ virus was found to be consistently sensitive and reproducible. A microplaque reduction method was utilized, in which two or fewer microplaques (cytopathic foci) constituted a positive result. Cytopathic change judged to be larger than a single microplaque, or cytopathic change of more than two microplaques was recorded as negative. The same end point titer was obtained in 36 replicate doubling dilutions of one serum using an estimated 25 $TCID_{50}$ virus from

three different stock virus reservoirs. The reproducibility, and sensitivity of the test was such that a two-fold increase in serum antibody titer was considered to be evidence of a seroconversion to the rhinovirus.

4. Plaque reduction neutralization test

The plaque reduction neutralization test as utilized in this study was not practical for application to large numbers of sera. The plaques produced were 2 mm and less in size. Microplaques, visible only with a microscopic examination, were inevitably present and the slightest defect in the monolayer could be confused with a plaque. Thus a microscopic examination was necessary for identification and enumeration of plaques.

5. Hyperimmune serum production

Immune serum was produced in rabbits against strains FS1-43 and VC-96. The titers achieved were very low (1/64).

Chickens failed to respond to inoculation with FS1-43 and VC-96 and remained without a neutralizing titer.

6. Serological relationship between FS1-43 and other bovine rhinovirus strains

Utilizing immune serum to rhinovirus strains FS1-43 and VC-96 the relationships between virus strains FS1-43, VC-96 and C-07 were compared. Serum to FS1-43 neutralized the homologous virus strain to a serum titer of 1/64. It neutralized strains VC-96 and C-07 to a serum titer of 1/16. Serum

to VC-96 neutralized the homologous virus strain to a serum titer of 1/64. It neutralized strains FS1-43 and C-07 to a serum titer of 1/16. Thus it is concluded that bovine rhinovirus strains FS1-43, C-07 and VC-96 are closely related but not identical.

FITC conjugated antibody to strains FS1-43 and VC-96 cross-fluoresced with cells infected with virus strains FS1-43, VC-96 and C-07.

7. Fluorescent labeled antibody examination of infected cells

A perinuclear ring and multiple small isolated cytoplasmic foci of specific fluorescence were observed in GBK cells infected with bovine rhinovirus, strain FS1-43, after incubation with a homologous fluorescent labeled antibody. As the infected cells became rounded and smaller the cytoplasm became intensely fluorescent.

D. Serology of Bovine Rhinovirus

1. Serological survey of clinically ill animals

Serum neutralization testing of acute and convalescent sera from 106 animals clinically ill with symptoms of bovine respiratory disease revealed that 70.8 percent of animals examined possessed antibodies to bovine rhinovirus (strain FS1-43). Samples examined were from 26 groups of animals representing over 5000 animals ranging in age from baby calves to mature cattle. The majority of animals were being fed for market.

A seroconversion, indicating a recent infection with bovine rhinovirus, was detected in 20.8 percent of animals examined. Table 6 contains serological data for herds and table 7 contains serological data for individual animals within each herd that seroconverted. Animals with a seroconversion to bovine rhinovirus were found in 15 of the 26 groups examined. Serological data obtained by Dr. M. L. Frey (20) from the same animals revealed ten of 15 of these groups contained animals that seroconverted to Mycoplasma agalactiae var bovis. Of the animals that seroconverted to bovine rhinovirus, 48 percent also seroconverted to M. agalactiae var bovis.

2. Specificity of antibody for the rhinovirus virion

Electron microscope examination of negatively stained virions following incubation with an excess quantity of homologous antibody revealed a specific binding of antibody to virions (figure 8). After antibody treatment the virions were contrasted in relationship to the other cellular debris present and thus easier to find and recognize as virions. No other cellular debris present in the preparation was bound with antibody to the bovine rhinovirus virion.

E. Animal Experimentation

1. Clinical observations

a. Group one After inoculation with bovine rhinovirus strain FS1-43 a yearling steer (400) developed very mild signs of a respiratory disease (table 8). The most indicative of a

Table 6. Summary of serological test by herd

Herd No.	No. of animals tested	No. with seroconversions to		
		RV[a]	MAB-4[b]	MAB-2[b]
1	1	1	0	0
2	2	1	1	1
3	1	0	0	0
4	4	0	2	1
5	5	1	1	1
6	6	1	5	0
7	4	0	0	0
8	7	1	1	0
9	7	3	5	0
10	2	0	2	0
11	6	1	0	0
12	8	1	1	0
13	9	1	8	1
14	4	1	0	1
15	3	0	0	0
16	6	0	0	0
17	5	0	4	0

[a]RV=Rhinovirus FS1-43, microplaque reduction serum neutralization test, seroconversion considered to be a two-fold increase in microplaque reduction titer

[b]MAB=*Mycoplasma agalactiae* var *bovis*, complement fixation test, four-fold and two-fold increases in titer

Table 6. (continued)

Herd No.	No. of animals tested	No. with seroconversions to		
		RV[a]	MAB-4[b]	MAB-2[b]
18	6	5	0	1
19	3	0	0	0
20	2	0	0	0
21	3	1	0	1
22	1	1	0	0
23	4	0	0	0
24	4	1	0	0
25	4	0	0	1
26	4	3	0	0
Total	111	23	30	8

Table 7. Summary of serological tests by individual animal

Herd No.	Seroconversion to RV[a]	Seroconversion to MAB-4[b]	Seroconversion to MAB-2[b]
1	+	-	-
2	-	-	+
	+	-	+
4	-	-	+
	-	+	
	-	+	
5	-	-	+
	+	+	
6	-	+	
	-	+	
	-	+	
	-	+	
	+	+	
8	+	+	
9	-	+	
	+	+	
	+	+	
	+	+	
	-	+	
10	-	+	
	-	+	
11	+	-	-

[a]RV=Rhinovirus FS1-43, microplaque reduction serum neutralization test, seroconversion considered to be a two-fold increase in microplaque reduction titer

[b]MAB=*Mycoplasma agalactiae* var *bovis*, complement fixation test, four-fold and two-fold increase in titer

Table 7. (continued)

Herd No.	Seroconversion to		
	RV[a]	MAB-4[b]	MAB-2[b]
12	+	-	-
	-	+	
13	+	+	
	-	+	
	-	+	
	-	+	
	-	+	
	ND	+	
	ND	+	
	-	+	
	-	-	+
14	+	-	+
17	-	+	
	-	+	
	-	+	
	ND	+	
18	+	-	
	+	-	
	+	-	+
	+	-	
	+	-	
21	+	-	+
22	+	-	
24	+	-	
25	-	-	+
26	+	-	
	+	-	
	+	-	

Figure 8. Bovine rhinovirus coated with a homologous antibody. Stained with phosphotungstic acid. 598,500X.

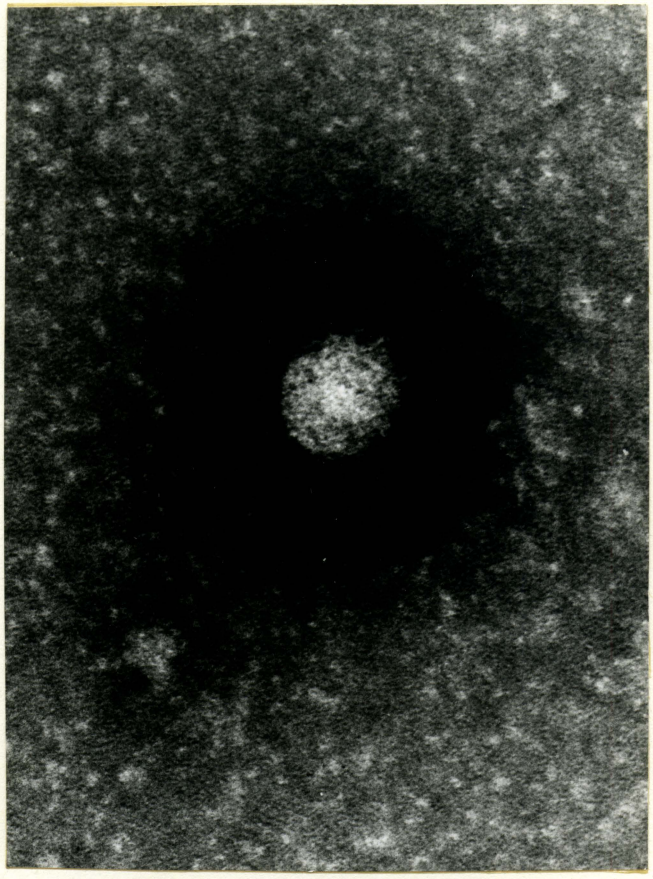


Table 8. Clinical response in calves exposed to rhinovirus strain FS1-43 and *Mycoplasma agalactiae* var *bovis*

Calf No.	Inoculum	Clinical response
Group 1		
400	RV[a]	Serous nasal discharge, reddened eyes, ocular discharge, increased respiration rate, reddened nasal mucosa
Group 2		
409	NTCF[b] contact control	Increased respiration rate, increased temperature, reddened nasal mucosa
410	RV	Reddened nasal mucosa
411	1. RV 2. Lung suspension	Reddened nasal mucosa Increased respiration rate, increased temperature, reddened nasal mucosa, dyspnea, anorexia
Group 3		
412	RV, MAB[c]	Serous to mucous nasal discharge, reddened nasal mucosa, ocular discharge, cough, increased respiration rate, increased temperature, dyspnea and anorexia
419	1. Contact 2. RV, MAB	Serous nasal discharge, reddened nasal mucosa, increased respiration rate, increased temperature, dyspnea, anorexia
418	MAB[c]	None
420	None	None
421	None	None

[a]RV=Rhinovirus strain FS1-43

[b]NTCF=Normal (noninfected) tissue culture fluid

[c]MAB=*Mycoplasma agalactiae* var *bovis*

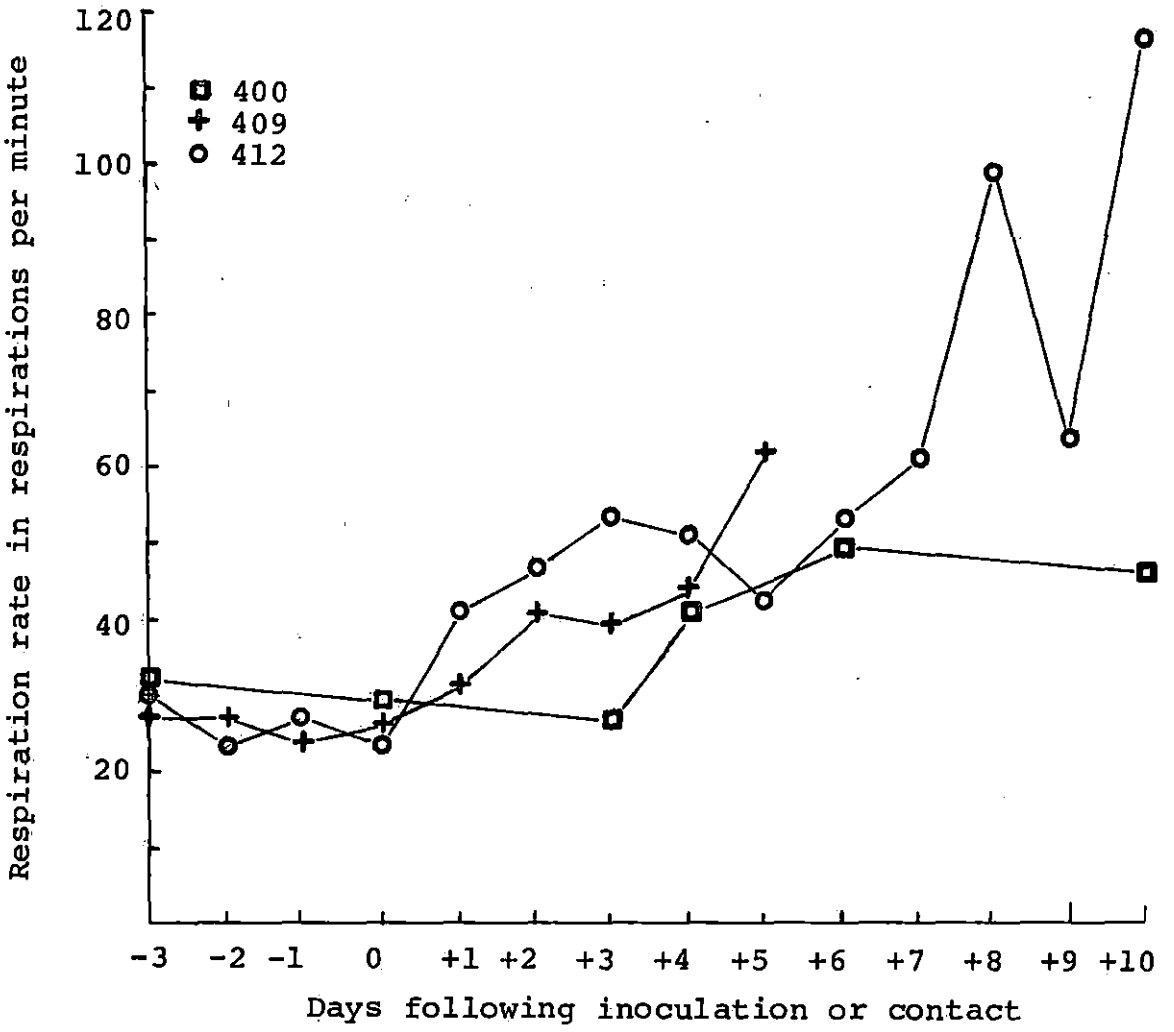
respiratory disease was the increase of the respiration rate from a pre-inoculation average of 27 respirations per minute (figure 9) to a post-inoculation rate of over 40 respirations per minute. All other observations were of a mild nature and could have been easily overlooked.

b. Group two Calves 410 and 411 did not exhibit clinical signs following intranasal and intratracheal inoculation with bovine rhinovirus strain FS1-43. The only finding was a reddening of the nasal mucosa.

Calf 409 (contact control) showed an increase in respiration rate (figure 9) and an increase in temperature to a high of 39.7 C.

Calf 411 remained asymptomatic for six days following intranasal and intratracheal inoculation with bovine rhinovirus strain FS1-43 except for a reddening of the nasal mucosa (see table 8 for clinical observations). After a subsequent intranasal and intratracheal inoculation with Mycoplasma spp. the temperature increased to a range of 39.4 to 39.7 C on days six to ten and the respiration rate increased with a high of 50 respirations per minute recorded on day nine. Respirations became labored and had associated rales. The animal had anorexia at the time of the elevations in temperature and respiration rate. Calf 411 was obviously ill from a respiratory disease.

Figure 9. Respiration rates of calves 400, 409 and 412 following infection. Calf 400 was inoculated with bovine rhinovirus strain FS1-43. Calf 409 was placed in contact with calves that were infected with bovine rhinovirus strain FS1-43. Calf 412 was inoculated with bovine rhinovirus strain FS1-43 and Mycoplasma agalactiae var bovis.


Respiration rate in respirations per minute
120
100
80
60
40
20
400
409
412
-3
-2
-1
0
+1
+2
+3
+4
+5
+6
+7
+8
+9
+10
Days following inoculation or contact

c. Group three Calves 412 and 419 became obviously ill following intranasal and intratracheal inoculation with bovine rhinovirus strain FS1-43 and Mycoplasma agalactiae var bovis. The temperatures (figure 10) increased to a peak on day eight of 40.6 and 40.8 C for calf 412 and 419, respectively. There was also an increase in respiration rate to peaks of 115 and 81, respectively (figure 9). Both calves were obviously ill and upon the slightest exertion demonstrated labored respiration with associated rales. Calf 412 exhibited a cough. Both animals showed anorexia from day six post-inoculation until necropsied.

Calf 418 after intranasal and intratracheal inoculation with Mycoplasma agalactiae var bovis remained asymptomatic until necropsied on day eight post-inoculation. All parameters used in this study remained at approximately the same levels as the averages of the pre-inoculation recordings. Figure 11 compares the temperature response of calf 418 (mycoplasma infection) with calf 411 (rhinovirus infection).

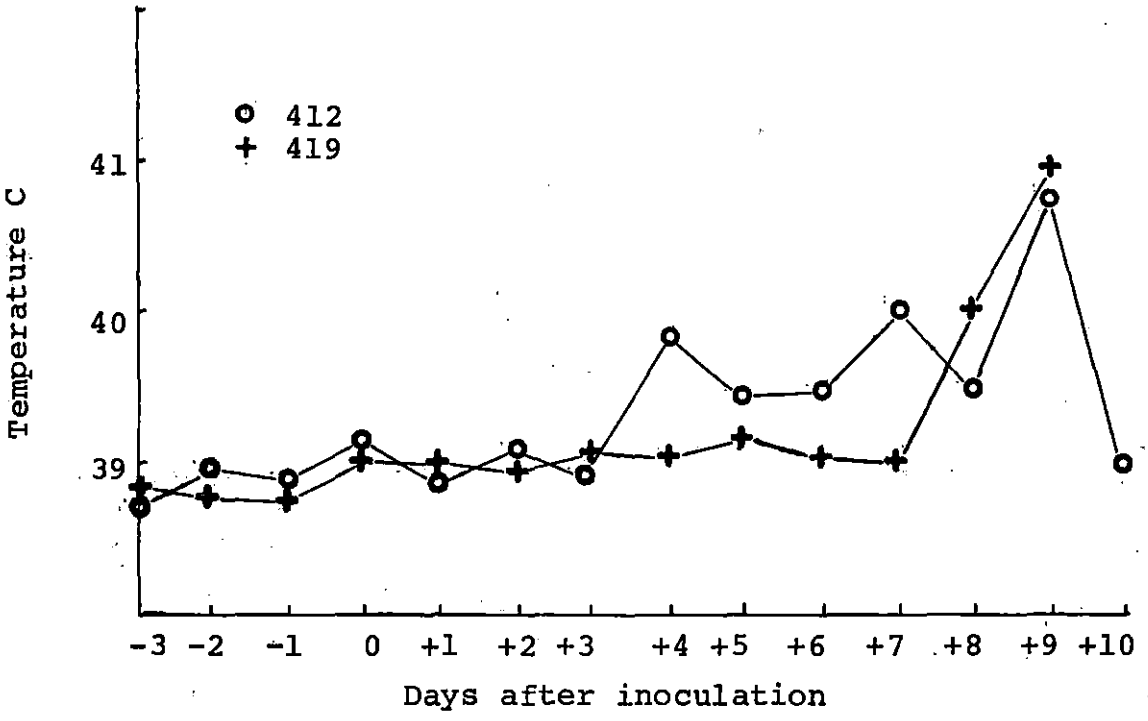
Calves 420 and 421 (control animals) did not exceed a temperature of 38.6 C or a respiration rate of 28 respirations per minute.

2. Virus recovery

Recovery of virus from calves is shown in table 9. Virus was not isolated from calves prior to inoculation. The virus was isolated from calves 400, 409 (contact control), 411, 412

Figure 10. Temperature response to bovine rhinovirus strain FS1-43 and Mycoplasma agalactiae var bovis in combination.

Figure 11. Temperature response after inoculation with bovine rhinovirus strain FS1-43 (411) and Mycoplasma agalactiae var bovis (418).


412
419
41
40
39
Temperature C
-3 -2 -1 0 +1 +2 +3 +4 +5 +6 +7 +8 +9 +10
Days after inoculation

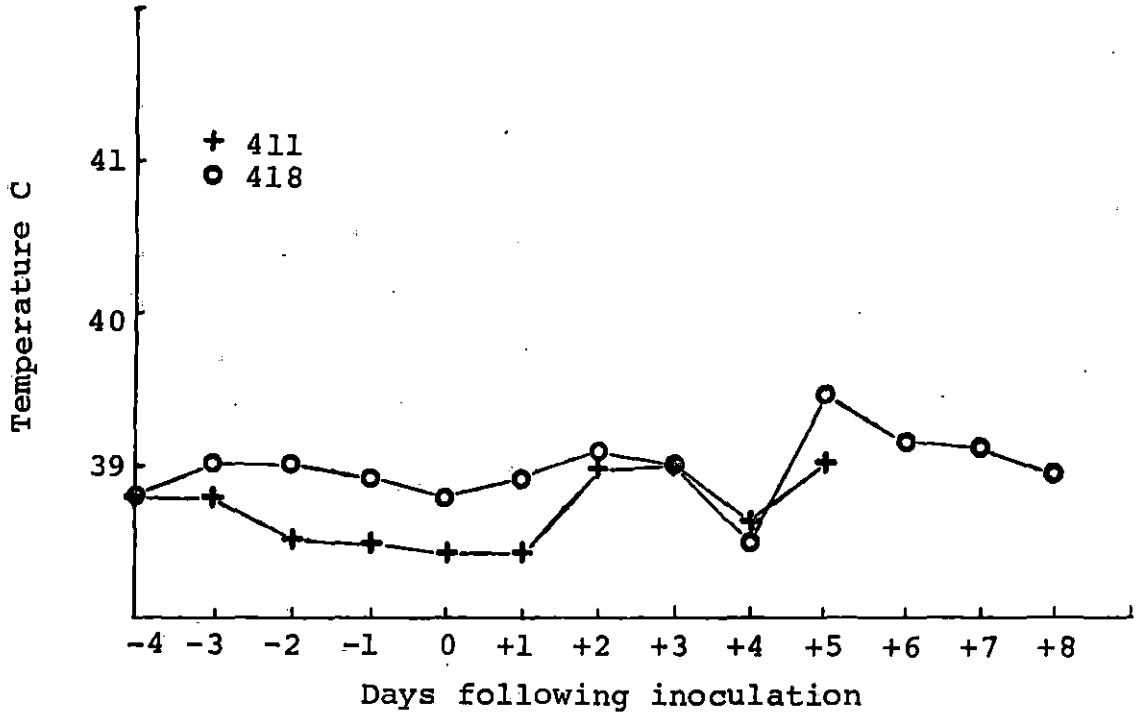

411
418
41
40
39
Temperature C
-4 -3 -2 -1 0 +1 +2 +3 +4 +5 +6 +7 +8
Days following inoculation

Table 9. Recovery of bovine rhinovirus from calves

Calf no.	Inoculum	Day of viral recovery										
		0	1	2	3	4	5	6	7	8	9	10
Group 1												
400	RV[a]	-[b]	-	+[c]	-	+	+	+	-	-		
Group 2												
409	Contact, NTCF[d]	-	-	-	+	+	-					
410	RV	-	-	-	-	-	-	-				
411	1. RV 2. Lung suspension	-	-	-	-	+	-	-				
Group 3												
412	RV, MAB[e]	-	-	-	-	-	-	-	+	+	-	-
418	MAB	-			-				-			
419	RV, MAB	-	-	-	-	-	+	+	+	+		
420	None	-					-					
421	None	-					-					

[a] RV=Bovine rhinovirus strain FS1-43

[b] - = No viral isolation

[c] + = Viral isolation

[d] NTCF=Normal (noninfected) tissue culture fluid

[e] MAB=*Mycoplasma agalactiae* var *bovis*

and 419. Calf 410 was negative for viral isolation on all attempts. Calves 418, 420 and 421 were negative for viral isolation throughout the study. The virus was readily isolated during the periods that the calves exhibited the most

extreme clinical symptoms. Virus could not be isolated from various tissues of calf 419 collected at necropsy.

3. Mycoplasma isolation

Mycoplasma spp. were not isolated from calves prior to experimentation (400 was not tested). Mycoplasma agalactiae var bovis was readily isolated from nasal swabs of calves 412, 418 and 419 following inoculation with reference strain Mycoplasma agalactiae var bovis. Calf 411 remained negative for Mycoplasma spp. following inoculation with pneumonic lung suspension containing organisms resembling M. agalactiae var bovis. M. agalactiae var bovis was isolated from the tarsal joint fluid of calf 419 at the time of necropsy.

4. Serological response to bovine rhinovirus strain FS1-43

The results are summarized in table 10. Animal 400 possessed a pre-inoculation titer (1/32) to bovine rhinovirus strain FS1-43. All other calves had a serum neutralization titer of less than 1:4. Calves 400, 411, 412 and 419 had seroconverted at the time the experiment was terminated. Calves 420, 421 and 418 remained sero-negative to bovine rhinovirus FS1-43. Post-inoculation serum neutralization tests were not performed on calves 409 and 410 because of the short duration of experimentation.

5. Gross pathological changes observed at necropsy

The gross pathological changes observed at necropsy are summarized in table 11. Calves 409 and 411 had lymph nodes

Table 10. Serological response to bovine rhinovirus FS1-43

Calf No.	Inoculum	Pre-inoculation titer[a]	Post-inoculation titer[a]	Post-inoculation day
Group 1				
400	RV[b]	32	128	(16)
Group 2				
409	NTCF[c], contact	<4	ND	
410	RV	<4	ND	
411	1. RV			
	2. Lung suspension	<4	8	(16)
Group 3				
412	RV, MAB[d]	<4	32	(10)
418	MAB	<4	<4	(8)
419	RV, MAB	<4	16	(8)
420	None	<4	<4	(15)
421	None	<4	<4	(15)

[a]Titer expressed as the reciprocal of the highest serum dilution that failed to neutralize 25 $TCID_{50}$ virus

[b]RV=Bovine rhinovirus strain FS1-43

[c]NTCF=Normal (noninfected) tissue culture fluid

[d]MAB=*Mycoplasma agalactiae* var *bovis*

that were enlarged and congested. Additionally several one to two cm areas of consolidation were observed on all surfaces of the lung. Calves 412 and 419 had multiple areas of emphysema in the lungs. The dorsal surface of the lungs of calf 419 was dusky purple in color. Other calves did not have any apparent gross pathological changes.

Table 11. Summary of gross lesions on necropsy

Calf No.	Inoculum	Gross lesions
Group 1		
400	RV[a]	None
Group 2		
409	NTCF[b], contact	Lymph nodes congested and enlarged, 1-2 cm areas of consolidation in lungs
410	RV	None
411	1. RV 2. Lung suspension	Lymph nodes congested and enlarged, 1-2 cm areas of consolidation in the lungs
Group 3		
412	RV, MAB[c]	Areas of emphysema in the lungs
418	MAB	None
419	RV, MAB	Areas of emphysema in the lungs, dusky purple areas on dorsal lung surface
420	None	None
421	None	None

[a]RV=Bovine rhinovirus strain FS1-43

[b]NTCF=Normal (noninfected) tissue culture fluid

[c]MAB=*Mycoplasma agalactiae* var *bovis*

6. *Histopathological findings*

Histologically, nasal mucosa and turbinates from rhinovirus infected calves, and from calves infected with rhinovirus and *Mycoplasma agalactiae* var *bovis*, were congested and had a few areas with micro-hemorrhages. The lamina propria had a minimal infiltration of lymphoreticular cells.

In calf 412 the serous glands of the nasal mucosa showed degenerative changes characterized by blurred cytoplasmic outlines and the nuclei were pycnotic with karyorrhexis. There was a perinuclear clearing of the cytoplasm. Edema around glandular tissue was evident but cellular infiltration was not observed. An area of exfoliated debris was found attached to a normal appearing epithelium. The trachea also exhibited edema of the lamina propria.

The lungs of all infected calves possessed peribronchial lymphoid aggregates. An impingement on bronchioles was observed in isolated areas. These areas were not seen in control calves.

Calves 409, 410 and 411 had bronchiolar hyperplasia resulting in an apparent occlusion of terminal bronchioles. The cells were swollen, had hydropic changes and had basophilic staining cytoplasm.

Lungs of all calves, infected and non-infected, showed focal areas of atelectasis and emphysema.

The lungs of calves 410, 411 and 419 had interstitial edema with some lymphoreticular infiltration. Affected areas had an associated congestion. The alveolar walls in focal areas were slightly thickened with mononuclear infiltration.

The lymph nodes associated with the respiratory tract in calves 409 and 411 were congested and had micro-hemorrhages.

The alveoli of all calves were free of exudate and cellular debris.

V. DISCUSSION

A multiplicity of viruses, bacteria, mycoplasmas and chlamydia (53) has been isolated from the nasal passages of cattle with respiratory diseases. Rhinoviruses are of current interest in relation to the common cold in man. At present, there have been few reports on isolations of bovine rhinoviruses (9,29,54,66,68). This is at least partially explained by the fact that rhinoviruses are extremely difficult to isolate and to maintain in culture. This report describes the characterization of a bovine rhinovirus isolated from a clinically ill animal in Iowa.

The properties described for the isolant FS1-43 fulfill current criteria for its designation as a bovine rhinovirus. The viral isolant FS1-43 is 26 to 32 nm in diameter, possesses RNA, is chloroform resistant and acid sensitive. The virus produced a cytopathic effect characteristic of rhinovirus and replicated in a narrow range of cells from the bovine.

Rhinovirus strain FS1-43 was found to be serologically related but not identical with strains VC-96 and C-07. Mohanty and Lillie (54) found strain C-07 to be related to SD-1 and Ide and Darbyshire (34) found that strain FS 3x was related to strains C-07, Sd-1 and 181/V. Betts et al. (5) found that strain EC11 was antigenically distinct from strain SD-1.

Isolant FS1-43 was found to replicate in bovine kidney cells (GBK and MDBK) as reported with other isolants (9,30,54, 68,83). Strain EC11 replicated in bovine kidney cells only after several passages in tracheal explants (66). Primary calf testicle cells were found refractory to rhinovirus replication as reported by Bogel and Bohm (9) but at variance to results obtained by Ide and Darbyshire (30).

High passage MDBK cells were found to be refractory to viral replication while low passage MDBK cells were found to support replication to titers comparable to other susceptible cells. Reed et al. (66) had observed replication in MDBK cells at a very low titer. This suggests a difference in cells obtained from different sources as observed by Ide and Darbyshire (30). Also a difference may exist in the ability of different strains to replicate readily in the cell type.

Replication of bovine rhinovirus strain FS1-43 was observed in fetal lung, EBTu and EBTu-B cells. The presence of very limited cytopathic changes within fetal lung and EBTu cells is similar to the effect observed in primary thyroid cells by Ide and Darbyshire (30). Subcultures in susceptible cells (GBK) revealed that the rhinovirus was replicating without exhibiting a corresponding degree of CPE. Attempts to adapt the virus to these cells were only partially successful.

No replication of bovine rhinovirus strain FS1-43 was detected in cell strains derived from fetal synovia, aortic intima or tracheal epithelium. Although this study has ex-

tended the number of cell types known to be susceptible to bovine rhinovirus, it is evident that the range is still very limited. This is comparable to the H type human rhinovirus.

Results indicated that in GBK cells there is no disadvantage with a stationary incubation of bovine rhinovirus, as was noted by Bogel (8). This is at variance with the reported findings of other investigators (30,68). It was necessary to rotate infected cultures of MDBK cells in order to obtain CPE although total viral yield was unaffected.

Incubation of cultures at 33 and 37 C did not produce the difference in final titer and progression of CPE as reported by several investigators (9,30,54,68,83). The probable explanation is that the inoculation of cell cultures in suspension, in this study, resulted in a more rapid adsorption rate, a more rapid progression of CPE and a high yield of virus at a earlier time as reported by Thomas, Conant and Hamparian (75). By achieving maximal titers and near maximal CPE early, the virus is not subjected to the same degree of thermoinactivation as is the case when monolayers of cells are inoculated. When cells are inoculated while in suspension, CPE is evidenced within 12 hours with inocula containing high concentrations of virus, and within 48 hours at the endpoint concentrations. This is considerably more rapid than the 18 hours to two weeks reported by Parsons and Tyrrell (58).

Bovine rhinovirus strain FS1-43 was found to replicate in bovine tracheal explants but did not affect ciliary activity as described (64,65,66).

Eosinophilic cytoplasmic masses in infected cells stained with hematoxylin and eosin corresponded to flame-red cytoplasmic masses in cells stained with acridine orange, as reported by Ide and Darbyshire (30,31). These masses were only observed in scattered cells. Fluorescent antibody stained cells revealed intracytoplasmic foci of specific fluorescence. Electron micrographs revealed large areas in the cytoplasm of infected cells which had vacuoles containing membrane bound cytoplasm-like material. Also large accumulations of ribosomes were observed. It is probable that the masses observed are accumulations of viroplasm. It would be expected that virions would be formed in these areas. The presence of virions could not be verified in this study even with the addition of magnesium (7).

Bovine rhinovirus strain FS1-43 produced plaques under an agarose overlay only after a minimum of three hours adsorption. This is considerably longer than the two hours reported by Ide and Darbyshire (34) which conforms with the two hours required for adsorption to a monolayer (75). The incubation temperature from 22 to 37 C during adsorption did not affect the results. Plaques produced by strain FS1-43 were smaller than rhinovirus plaques produced by Ide and Darbyshire (34).

The addition of 1 M $MgCl_2$ resulted in a two- to four-fold increase in plaque diameter.

Bovine rhinovirus strain FS1-43 was thermoinactivated after 12 and eight hours at 34 and 37 C, respectively, at a rate in agreement with Ide and Darbyshire (31). However, at 22 and 4 C this isolant was more rapidly inactivated than strain RS 3x, as reported by Ide and Darbyshire (31). Rosenquist reported a marked variability in the rate of thermoinactivation between four rhinovirus isolants.

The loss of approximately 90 percent of infectivity in 50 days at -70 C is at extreme variance with RS 3x isolant which did not lose infectivity titer after one year at -70 C (31).

Strain FS1-43 was not stabilized against thermoinactivation at 50 or 57 C with 1 M $MgCl_2$ as was strain C-07 (54). This agrees with findings on strains Sd-1, 181/V and RS 3x (31) which were not stabilized.

Strain FS1-43 did not hemagglutinate erythrocytes under the conditions utilized. The complement fixation test did not detect known positive serum to bovine rhinovirus strain FS1-43. This is probably due to low virus yield as observed by Bogel (8).

Most serology with bovine rhinovirus has been done with tube serum neutralization tests. This study also used the serum neutralization test but as adapted to a micro test. The microplaque reduction serum neutralization test provided the

advantages of economy of virus, serum, titration plates, physical facilities and time. Test results could be obtained in 48 hours compared with a minimum of 24 hours to produce a dense monolayer of cells and an additional 72 hours to obtain test results with the plaque reduction neutralization test.

Plaques produced in the plaque reduction neutralization test were small enough that identification and enumeration required microscopic examination thus negating the usual advantage of plaque tests. Titers obtained at 50 and 70 percent plaque reduction paralleled those obtained by the microplaque reduction serum neutralization test but were consistently higher by a six- and four-fold factor, respectively.

The demonstrated reproducibility and sensitivity of the microplaque reduction method of serum neutralization was such that a two-fold increase in serum titer was considered as a seroconversion to bovine rhinovirus. Replicate samples titrated in the plaque reduction neutralization test added credence to the reliance on a two-fold microplaque reduction qualifying as a seroconversion to an infection.

A somewhat lower percentage of animals demonstrated antibodies to bovine rhinovirus (70.8 percent) than had been previously reported. This finding is influenced by the fact that serum samples were primarily from young cattle which Ide and Darbyshire (33) found to have lower titers than animals of increased age.

Ruhnke (69) found that 90.6 percent of pneumonic cattle lungs were positive for Mycoplasma spp. Comparison of serological data for Mycoplasma spp. (20) with serological data for rhinovirus on the same animals found that 48 percent of cattle that seroconverted to rhinovirus also seroconverted to Mycoplasma agalactiae var bovis. A two-fold increase in serum titer was regarded as a seroconversion to M. agalactiae var bovis because of the slow appearance of antibodies to certain mycoplasmas in infected cattle (19).

Several workers have failed to produce signs of respiratory disease in calves inoculated with bovine rhinovirus (9, 32,49,83). Betts et al. (5) and Mohanty et al. (55) demonstrated clinical signs of respiratory disease but concluded that the animals were not obviously ill and a diseased condition would not be detected under ordinary conditions. The present study gave similar results.

Rosenquist's isolants (68) were obtained from clinically ill animals as the only recognized pathogens by either isolation or serology. Rosenquist did not report attempted mycoplasma isolation or serology. Strain FS1-43 was isolated from a clinically ill animal. Mycoplasmas were isolated from the same animal as bovine rhinovirus strain FS1-43 (20). No other pathogenic organisms were detected or suspected as a result of the isolation and serological procedures utilized.

Intranasal and intratracheal inoculation of a calf with Mycoplasma agalactiae var bovis did not produce clinical signs within eight days.

Bovine rhinovirus strain FS1-43 and Mycoplasma agalactiae var bovis inoculated intranasally and intratracheally into calves produced obvious clinical signs characterized by increased temperature (to 40.8 C), increased respiration rate (to 115 respirations per minute), slight nasal discharge, reddened nasal mucosa, cough, dyspnea and anorexia.

These findings add support in vivo to the findings by Reed (64) that rhinovirus infection provides a more favorable environment for growth of mycoplasmas. This was suggested as one possible mechanism of the total pathogenic effect of the rhinovirus and mycoplasma.

Macroscopic pathological changes observed at necropsy were minor and could readily have been discounted in agreement with reported findings (5,32). Mohanty et al. (55) reported more serious macroscopic changes in the lungs of infected cattle.

Virus was isolated from nasal swabs of five of six animals infected with FS1-43 including a contact control. Virus was not isolated from nasal epithelium, turbinates, tonsils or lung. Similar results were obtained by Bogel and Bohm (9) and Mohanty et al. (55) but Betts et al. (5) recovered strain EC11 from turbinate, tonsil and trachea of infected calves.

The calves did respond serologically to infection with FS1-43.

All of the histopathological changes observed in experimentally infected calves were minimal and could have been regarded as insignificant in routine histological examinations.

The focal necrosis of turbinate epithelium and changes in epithelium from psuedo-stratified columnar ciliated to low cuboidal non-ciliated cells as reported by Ide and Darbyshire (32) was not observed. Betts et al. (5) observed changes in the glands of the turbinates similar to those observed in calf 412. It is important to note that rhinovirus strain FS1-43 utilized in this study did not produce cessation of ciliary activity in organ cultures of tracheal explants while strains RS 3x and EC11 utilized by Ide and Darbyshire (32) and Betts et al. did cause a cessation of ciliary activity in organ culture.

Marked peribronchiolar lymphoid aggregates and masses of monocytes observed by Mohanty et al. (55), as well as other changes described, were not comparable to the minor changes observed in this study.

VI. SUMMARY

The isolant FS1-43 was characterized as a bovine rhinovirus. The virus was antigenically related to bovine rhinovirus strains VC-96, C-07, 181/V, RS 3x and Sd-1.

Strain FS1-43 was found to replicate in GBK, low passage MDBK, EBTu, EBTu-B, and fetal lung cells. The virus replicated in tracheal explants but did not stop ciliary action.

Rotation and temperature (either 33 or 37 C) did not increase the yield of virus or progression of CPE in GBK cells. MDBK cells required rotation before CPE was exhibited. EBTu and fetal lung cells did not exhibit CPE in correlation with the replication of virus.

Plaques were produced under agarose overlay after three hours adsorption. The addition of 30 mM $MgCl_2$ to the overlay enhanced the size of plaques produced by two- to four-fold.

FS1-43 was more rapidly thermoinactivated at 22, 4 and -70 C than strain RS 3x. The virus was not stabilized against thermoinactivation by 1 M $MgCl_2$.

FS1-43 did not react in hemagglutination and complement fixation tests. Comparable results were obtained utilizing a microplaque reduction serum neutralization test and the plaque reduction neutralization test.

In a serological survey of Iowa cattle clinically ill with BRD, 70.8 percent of the sampled cattle had serum antibodies to bovine rhinovirus. Serological evidence revealed

that 20.8 percent of animals examined had been recently infected with bovine rhinovirus FS1-43. Serological evidence also revealed that 48 percent of these animals also had been recently infected with Mycoplasma agalactiae var bovis.

After inoculation with bovine rhinovirus strain FS1-43 calves (three of three) exhibited mild clinical signs of which an increased respiration rate was the most noticeable. After inoculation with Mycoplasma agalactiae var bovis a calf (one of one) did not exhibit clinical signs. After inoculation with bovine rhinovirus strain FS1-43 and Mycoplasma agalactiae var bovis calves (three of three) exhibited obvious clinical signs characterized by increased respiration rate, increased temperature, reddened nasal mucosa, dyspnea and anorexia.

The nasal mucosae and turbinates of infected calves were congested grossly and histologically.

Minimal pathological changes were noted. No difference was noted between the changes observed in calves monoinfected with rhinovirus and those infected with rhinovirus and Mycoplasma agalactiae var bovis.

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VIII. ACKNOWLEDGMENTS

The author wishes to express his appreciation for the guidance and encouragement given by Dr. M. L. Frey during this study. The time and advice of my committee members, Dr. R. A. Ball, Dr. C. J. Maré and Dr. D. C. Beitz, is gratefully acknowledged.

Thanks are expressed for the assistance and cooperation of the staff of the Veterinary Medical Research Institute throughout these studies. The author wishes to acknowledge the following who offered special and invaluable assistance: Dr. M. H. Smith, Pat Hale, Mrs. Ellen Osheim, Howard Lehmkuhl, Cliff Annis, Al Ritchie and Mrs. Ione Vold.

Special thanks are due to my wife Nancy and my children Max, Russell and Martina for their patience, encouragement and unfailing support.

This project was supported in part by a grant to Iowa State University from Jensen-Salsbery Laboratories, Division of Richardson-Merrell Inc.