

## ISOLATION AND IDENTIFICATION OF A BOVINE RESPIRATORY SYNCYTIAL VIRUS IN CZECHOSLOVAKIA

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### Abstract

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A bovine respiratory syncytial virus (RSV) was isolated in primary foetal bovine kidney cell cultures from an outbreak of acute respiratory disease in a herd of feedlot cattle. The cytopathic changes developed slowly with large multinucleated syncytia being regularly observed only after 5 to 7 passages. The isolate was identified by virus-neutralization and immunofluorescence tests and demonstrated by electron microscopy. Of three serologically negative calves infected experimentally with the virus, two developed mild respiratory disease and all three animals had specific antibodies in their sera.

*Bovine respiratory syncytial virus, feedlot cattle, isolation, identification, experimental infection, calf.*

The involvement of bovine respiratory syncytial virus (RSV) in the development of acute respiratory disease in cattle was demonstrated a few years ago. Dogget et al. (1968), having found neutralizing antibody against human RSV in bovine serum, suggested that a virus antigenically similar to the human RSV was present in cattle. The first isolations of RSV were reported by Paccaud and Jacquier (1970) in Switzerland and by Inaba et al. (1970) in Japan. Since then, bovine RSV has been isolated in many countries and its involvement in the development of acute respiratory disease was demonstrated in both calves and adult cattle (Jacobs and Edington 1971; Smith et al. 1975; Köves and Bartha 1975; Wellemans and Leunen 1975; Bitch et al. 1976).

The present report describes the isolation of a bovine RSV that was responsible for an outbreak of acute respiratory disease in feedlot cattle of one farm in Czechoslovakia at the beginning of 1976.

### Materials and Methods

#### Epizootic Data

In January 1976 an acute respiratory disease was recorded in the feedlot of farm ND, affecting all 48 feedlot cattle. The course of the disease was severe; one heifer died and another was emergency slaughtered in the acute stage of illness. No cattle became ill on the farm itself that was about 500 m distant from the feedlot.

#### Collection of Specimens

Nasal and ocular secretion material for virological examination was collected with sterile cotton swabs and placed in 2 ml of Earle's maintenance medium containing antibiotics. These samples were frozen at  $-70^{\circ}\text{C}$  until examined. Blood samples for serological examination were taken at the same time.

Nasal and tracheal mucosa, lung, pulmonary lymph node, liver and spleen specimens were taken from the emergency slaughtered heifer and made into 10 per cent suspensions immediately after collection. These suspensions were inoculated into cell cultures without previous freezing. Specimens from the respiratory tract were also taken for immunofluorescent examination.

### Virus Isolation

Virus isolations were attempted in primary foetal bovine kidney (FBK) cell cultures grown in Hank's medium containing 10 per cent foetal calf serum at 37 °C. The tube cell cultures were infected with 0.2 ml of organ suspension or with the same volume of nasal or ocular swab material, with the infection being made either directly into the medium of growing cells several hours after the culture was started or after the growth had advanced to the formation of a monolayer. In the former case the medium was merely replaced with Earle's serum-free medium as soon as the monolayer was formed. In the latter case the infective material was allowed to be adsorbed for 30 minutes after inoculation into the monolayer, and Earle's maintenance medium was then added to make 1 ml. All the cell cultures were then incubated for 7 to 9 days, being daily observed for CPE or non-specific degeneration. If a CPE was noted, the cultures were frozen and employed for further passage. If no CPE was observed, the samples were discarded after five additional passages.

### Virus Identification

The isolate was identified by the cross neutralization test using specific rabbit antiserum against bovine RSV strain BF 3\* obtained through the courtesy of Dr Köves and Dr Bartha of the Veterinary Research Institute, Budapest, Hungary. Antisera against parainfluenza-3 (PI-3) strain T<sub>1</sub>/59\* and against infectious bovine rhinotracheitis (IBR) strain R-6\* were used as controls.

### Serological Examination

Virus-neutralizing antibodies in the blood sera of experimentally infected calves were determined by the micromethod using Linbro IS-FB-96 plates. Two serial twofold dilutions of each serum were incubated in 0.05 ml volumes with the same volumes of viral suspension containing 100 TCID<sub>50</sub> of virus. After 1 hour incubation at room temperature a suspension of cells of an established bovine foetal trachea cell line in Earle's minimal essential medium with 10 per cent foetal bovine serum was added in the quantity of 0.05 ml (200 000 cells per ml). The plates with polystyrene covers were incubated at 37 °C in an atmosphere of 5 per cent CO<sub>2</sub> in air and the results were read 4 to 5 days after infection. The serum titres were given as the reciprocal of the highest serum dilution causing complete inhibition of CPE in at least one of two wells.

### Immunofluorescent and Cytological Examination

Primary cell cultures of FBK grown on glass slides in test tubes were washed with buffered saline at 24, 36, 48, 72 and 96 hours after infection with the isolate in its 6th to 8th passage, air-dried and fixed in chilled acetone for 10 minutes. The isolate was identified by the indirect immunofluorescence technique using rabbit antiserum against bovine RSV, strain BF 3, and swine anti-rabbit/FITC conjugate (Institute of Sera and Vaccines, Prague). Conjugates against PI-3 and IBR viruses, produced by Bioveta, Nitra, were used as controls using the direct immunofluorescence technique. The same conjugates were employed to demonstration of PI-3 and IBR viruses in cryostat sections of the respiratory tract from the emergency slaughtered heifer (Pospíšil et al. 1972, 1975).

For cytological examination, infected cell cultures were fixed in Bouin's fluid and stained with haematoxylin and eosin.

### Electron Microscopy

A primary cell culture of FBK infected with the RSV isolate in its 8th passage was fixed in 1 per cent glutaraldehyde six days after infection. The cells were then scraped from the glass, pelleted, postfixed in 1 per cent OsO<sub>4</sub>, dehydrated in acetone and embedded in Araldite M. Ultrathin sections were cut on a Tesla BS 490 ultramicrotome, stained with lead citrate and uranyl-acetate and examined with a Tesla BS 513 electron microscope.

### Haemadsorption and Haemagglutination Test

Haemadsorption of the isolate was tested with 1 per cent suspensions of bovine, guinea-pig, sheep, rabbit and fowl erythrocytes according to Vogel and Shelekov (1957). Haemagglutinating activity of the isolate was tested with 0.5 per cent suspensions of three times washed erythrocytes specified above. Serial twofold dilutions of the isolate were mixed with equal volumes of the suspension of erythrocytes and the result was read after 60 min. at room temperature or 24 hours at +4 °C.

\* Available from the Collection of Animal Pathogenic Microorganisms, 62132 Brno, Czechoslovakia.

## Experimental Infection

Three calves approximately three weeks old that originated from a farm having no history of respiratory infection and proved negative when examined serologically by the virus-neutralization test against RSV were employed for experimental infectivity studies. They were infected intranasally with 5 ml and intratracheally with 10 ml of infectious tissue culture fluid containing  $10^8$  TCID<sub>50</sub>/0.1 ml virus in the 9th passage. The calves were examined daily for signs of clinical illness and blood-sampled weekly for serological examination.

## Results

### Clinical and Pathoanatomical Findings

The outbreak of respiratory disease in the feedlot of farm ND was characterized by a sudden onset of disease in all 48 feedlot cattle. The clinical signs included a rise in body temperature to about 41 °C, serous nasal discharge, paroxysms of cough, inappetence and general malaise. In two to three days the nasal discharge changed from serous to mucous or mucopurulent, and marked dyspnoea developed. One heifer died and another was emergency slaughtered in the acute stage of illness. The remaining animals showed gradual subsidence of the symptoms in 7 to 10 days, but were slaughtered, since most of them had reached slaughter weight before becoming ill and were losing weight because of inappetence.

Pathoanatomical examination of the heifer that died and of the emergency slaughtered animal demonstrated pulmonary oedema and emphysema, lobular bronchopneumonia and marked hyperaemia of the nasal and tracheal mucosa with mucous to mucopurulent exudate.

### Virus Isolation

In the first two passages no morphological changes were observed either in primary FBK cell cultures infected several hours after the culture was started or in those infected after the growth had advanced to the formation of a monolayer. In the 3rd passage solitary cells with a refractory granular cytoplasm appeared 9 days after infection and detached and floated in the medium two days later. However, the morphological changes in this and subsequent passage were produced only by two specimens (organ suspension from the lung of the emergency slaughtered heifer and nasal swab material derived from a steer) if the infecting material was inoculated shortly after the culture was started. On the other hand, no morphological changes were produced by the other infecting materials in any passage or if inoculation was made into a FBK monolayer.

In the 4th and 5th passages scattered refractory cells with a granular cytoplasm were seen in the cell cultures 7 days after infection, giving rise to small syncytia later on. By 8 to 9 days after infection some cells clumped up and small holes appeared so that the cytopathic effect (CPE) became more distinct. This development of CPE was seen mainly in cell cultures infected with the isolate from the lung, whereas the CPE seen after infection with the nasal swab material was less distinct. The other cell cultures infected with nasal or ocular swab materials showed no changes and were therefore discarded after the 5th passage.

In the 6th to 8th passages the interval between infection and the appearance of CPE was only 5 days and distinct syncytia were observed in cell cultures infected with the isolate from the lung (Plate III, Fig. 1). On the other hand, cell cultures infected with the nasal swab material that had produced a slight CPE in the preceding passages showed hardly any changes in the 6th passage and no CPE in the 7th and 8th passages; this specimen was therefore discarded.

In the 9th and subsequent passages of the isolate from the lung, changes were first observed as a rule 4 days after infection. They consisted of syncytium formation, clumping and detaching of the cells and the appearance of holes in the cell sheet. The syncytia ranged from formations containing only a few nuclei to multinucleated ones containing more than 50 nuclei (Plate III, Fig. 2). In the cytoplasm of these cells eosinophile inclusions were often present.

In spite of the extensive changes produced in cell cultures the highest titre yielded by the virus was only  $10^3 - 10^{3.5}$  TCID<sub>50</sub>/0.1 ml and could not be increased by either cultivation on a roller or incubation at various temperatures.

### Virus Identification

The virus was identified by the cross neutralization test using specific rabbit antiserum against bovine RSV strain BF 3. The isolate was completely neutralized by this rabbit serum, whereas antisera against PI-3 and IBR virus had no virus-neutralizing effect (Table 1).

Table 1  
Serological identification of the isolated virus

Strain	Antiserum		
	bovine RSV-BF 3	PI-3	IBR
Isolate from the lung	1 : 64	—	—
Bovine RSV-BF 3	1 : 64	—	—

### Immunofluorescent Examination

When examined by indirect immunofluorescence, the primary FBK cell cultures infected with the isolate from the lung showed specific fluorescence in the cytoplasm of the infected cells. In the 6th passage where the isolate was examined by immunofluorescence for the first time, specific fluorescence was seen in the form of granular perinuclear fluorescence 36 hours after infection (Plate IV, Fig. 3). Examination at later intervals revealed a progressive rise in the number of specifically fluorescent granules, with granular fluorescence being shown by the entire cytoplasm by 96 hours. In the 8th passage, specific fluorescence was first observed 24 hours after infection and fluorescent granules were distributed throughout the cytoplasm by 72 hours (Plate IV, Fig. 4). The nuclei were invariably negative.

Examination of BFK cell cultures infected with the material derived from the respiratory tract of the emergency slaughtered heifer and of cryostat sections from the lung of this animal using conjugates against the PI-3 and IBR viruses yielded negative results.

### Electron microscopy

Ultrathin sections of infected cells showed viral particles in the process of maturation by budding from the plasma membrane (Plate V, Fig. 5). Two particle forms were observed: spherical particles and filamentous forms varying considerably in length. Both forms were about  $130 \mu\text{m}$  in diameter and showed a distinct unit membrane structure of the envelope with spikes projecting from the surface.

The spherical particles contained electron dense dots, whereas the inner structure of the filamentous particles was not well defined and seemed to consist of tubules or filaments (Plate IV, Fig. 6).

### Haemadsorption and Haemagglutination Test

The isolate from the lung of the emergency slaughtered heifer neither adsorbed nor haemagglutinated erythrocytes of any of the species employed.

### Experimental Infection

Two of 3 serologically negative calves (Nos. 1, 2 and 3) infected with the isolate from the lung of the emergency slaughtered heifer developed mild, but distinct clinical signs of respiratory disease. The first symptoms consisted of a temperature rise to 39.6–39.9 °C, serous nasal discharge and increased respiratory rate and were observed on the 5th day after infection. On day 6, calf No. 3 showed a further rise in body temperature to 40.1 °C accompanied by transient inappetence and general malaise, whereas the body temperature of calf No. 2 was below 40 °C. On days 7 and 8 the body temperatures of the two calves returned to normal. Nasal discharge disappeared on day 9. Calf No. 1 showed no signs of clinical disease at all.

Specific serum antibodies were demonstrated in calf No. 2 at 14 days after infection in a titre of 1:6 and in calves Nos. 1 and 3 as late as 3 weeks after infection in titres of 1:16 and 1:32, respectively. At 28 days after infection the serum antibody titre of calf No. 1 was 1:32 and those of calves Nos. 2 and 3 were 1:64.

### Discussion

From the results reported here it appears that bovine RSV is one of agents responsible for the development of the respiratory syndrome in young cattle in Czechoslovakia. In this study it was isolated from an emergency slaughtered heifer during an outbreak of severe acute respiratory disease in a herd of feedlot cattle.

The isolation and cultivation attempts were successful only in cell cultures that were infected with the virus directly into the medium of growing cells several hours after the culture was started, whereas attempts to isolate the virus from the same material after inoculation into the monolayer failed. Cytopathic changes were absent in the first two passages, developed slowly in subsequent ones and from the 9th passage onwards were regularly seen as early as 4 to 5 days after infection. The typical manifestation of the infection of cell cultures was formation of multinucleated syncytia. Inhibition of the CPE of our isolate by specific anti-serum against the reference strain of bovine RSV in the virus-neutralization test demonstrated that the two strains are antigenically identical and that the isolate is bovine RSV. The virus was also demonstrated by electron microscopy and identified by immunofluorescence.

The titre of the virus ranged from  $10^3$  to  $10^{3.5}$  TCID<sub>50</sub>/0.1 ml and could not be increased by temporary cultivation of the cells on a roller. The problem of low RSV titres was also discussed by Smith et al. (1975) who having found as Paccaud and Jacquier (1970) did in their filtration studies that much of the

infectivity of RSV was retained by a  $0.45 \mu$  filter, suggested that their failure to obtain a high titre of virus may have been due to virus aggregation.

Although the isolation of bovine RSV presents some difficulty and the virus was therefore isolated rather rarely to date, its involvement in the development of respiratory disease in cattle is considered to be high. The evidence is based on the observations that antibody titres against bovine RSV increased after outbreaks of respiratory disease in various localities. Mohanty et al. (1976) drew attention to considerable sensitivity of RSV to external environment and recommended that in virus recovery attempts infectious material be inoculated into susceptible cells within one hour after collection, without previous freezing and thawing. Wellemans (1977) suggested that the difficulty in isolating this virus was due to rapid antibody formation and subsequent virus neutralization in organ suspensions and recommended mainly immunofluorescent examination of ultrathin cryostat sections for rapid diagnosis.

It is of interest to note that in spite of its high pathogenicity bovine RSV generally produces only moderate or even subclinical disease in experimentally infected calves. Smith et al. (1975) in their study on a bovine RSV concluded that the virus produced clinical disease particularly in calves with humoral antibody in agreement with observations reported in human medicine. Mohanty et al. (1976), on the other hand, found no evidence to indicate that the development of clinical symptoms depended on the presence of serum antibodies and concluded that in this respect bovine RSV differs from human RSV. Another difference between human RSV and bovine RSV infections were pointed out by Paccaud and Jacquier (1970) who found that although the clinical signs in cattle were very similar to those described in man, the most severely affected animals were cows and not calves in contrast to man where the course of the disease is usually more severe in infants than in older children or adults. In the population of cattle followed in the present study the course of the disease was also most severe in the heaviest animals.

In view of the acute form of the disease reported here we had to take into account other possible causative agents such as PI-3 and IBR viruses that have often been responsible for respiratory disease of cattle in our country. Even though the herd of feedlot cattle from which our isolate had been obtained was soon liquidated and we have therefore no paired serum samples to provide unequivocal evidence for the aetiological role of our bovine RSV isolate, the cultural findings and the results of immunofluorescent examination suggest that this virus was the only cause of the disease and that the involvement of PI-3, IBR or other viruses can be ruled out. The cryostat sections from the emergency slaughtered heifer showed no specific fluorescence with conjugates against the PI-3 and IBR viruses. Moreover, it seems reasonable to assume that if infection with a virus other than bovine RSV had been present, the agent would have been isolated, since the CPE produced by bovine RSV developed very slowly and required many passages. In "acute" serum samples, antibodies against IBR virus were not demonstrated at all and antibodies against PI-3 virus were detected in titres of 1 : 32 to 1 : 256. Since these "acute" sera were collected at the beginning of clinical illness, they contained no detectable antibodies against bovine RSV either.

However, the results of preliminary serological examinations for antibody against bovine RSV in several herds in our country can be regarded as suggestive evidence of its rather frequent incidence. Attention should therefore be directed to RSV particularly in those cases where acute respiratory disease affects both

adult cattle and calves and often subsides without its aetiology being disclosed. To combat such outbreaks of respiratory disease further studies on the pathogenesis of RSV infection are needed to provide more information on the factors underlying the development of clinical disease particularly with respect to the role of humoral antibodies.

### **Izolace a identifikace bovinního respiračního syncytiálního viru v ČSSR**

Z akutního respiračního onemocnění žírného skotu byl na primárních buněčných kulturách fetálních bovinních ledvin izolován bovinní respirační syncytiální virus (RSV). Rozvoj cytopatických změn postupoval pomalu a teprve v 6. až 8. pasáži se pravidelně vytvářela velká mnohojaderná syncytia. Virus byl identifikován virusneutralizačním a imunofluorescenčním testem se specifickým králičím antisérem a byl prokázán v elektronovém mikroskopu. U 2 ze 3 serologicky negativních telat se po experimentální infekci vyvinulo poměrně mírné respirační onemocnění a u všech 3 telat byla prokázána tvorba specifických protilátek.

### **Изоляция и идентификация синцитиального вируса крупного рогатого скота в ЧССР**

Из острого дыхательного заболевания жирного скота на первичных культурах почек зародышей крупного рогатого скота был изолирован респираторный синцитиальный вирус крупного рогатого скота (РСВ). Развитие цитопатических изменений протекало медленно, лишь в 6—8 пассажах регулярно образовались крупные многоядерные синцитии. Вирус был определен нейтритализационным и иммунофлуоресцентным тестами со специфической кроличьей антисывороткой и был выявлен в электронном микроскопе. В двух случаях из трех серонегативных телят после экспериментальной инфекции возникло сравнительное небольшое дыхательное заболевание и во всех трех случаях было установлено образование специфических антител.

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