

**ISOLATION AND CHARACTERIZATION OF A
BOVINE HERPESVIRUS 3 FROM CASES OF
CALF PNEUMONIA IN IRAN**

By

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SUMMARY

A virus strain was isolated from 1 to 3 month - old calves in a herd suffering from sever respiratory disease including pneumonia.

The virus possessed cytopathogenicity for bovine foetal kidney cell culture producing Cowdry's type A intranuclear inclusions in infected cells.

The virus particles had characteristic herpesvirus features in negatively stained electron microscopic preparations and measured approximately 170-350 nm. and 100 - 110 nm. with and without envelope, respectively.

The isolate was sensitive to ether and chloroform, did not agglutinate guinea pig erythrocytes and did not show heamadsorption of guinea pig erythrocytes to infected tissue culture cells.

Cross neutralization tests indicated that the virus was serologically distinct from bovine herpesvirus 1, bovine herpesvirus 2 , and parainfluenza 3 viruses. The virus, finally , was indentified as bovine herpesvirus 3 and was designated as Razi 492 strain.

The Razi 492 bovine herpesvirus 3 produced typical respiratory disease in calves when they were exposed by the intratracheal and intranasal routes. The inoculated calves showed pyrexia, increase in respiratory rate, coughing,

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dispnea, slight anorexia, depression, nasal discharge accompanied by consolidation of the lungs .

The virus was unable to raise a demonstrable level of neutralizing antibody in the inoculated calves but animals recovered from infection seemed to be immune to reinfection.

Serological response of rabbits against the virus was also weak. Neutralizing antibody, however, was produced in this animal by 15 weekly interval intravenous inoculations of the virus.

INTRODUCTION

During the past 7 years pneumonia has been considered as one of the most important clinical signs of 1 to 4 months old calves in several large breeding and dairy farms around Tehran. In cases suffering severely, both acute and chronic phases of the disease has been observed. The infected calves either died or were urgently slaughtered.

In less severe cases the recovery rate has been higher specially when broad-spectrum antibiotics were used for treatment soon after the appearance of symptoms and the management of the flock was improved.

No marked seasonal incidence has been reported although losses has frequently occurred during the cold seasons (late fall and winter) in farms where calves have been kept intensively under bad sanitary conditions.

Various microorganisms have been postulated as the causative agents of the disease but only staphylococcus, streptococcus, corynebacterium, coliforms and pasteurilla were recovered from the sick animals. Recently, however, a virus was isolated from calves in severely infected herds. Some of the biological characteristics of this virus and the possibility of its primary role in producing calf pneumonia was investigated and the results are reported in this communication.

MATERIALS AND METHODS

Source of materials - In November 1977 a severe pneumo - enteritis was reported among 1 to 4 months old calves at a large industrialized dairy farm in Tehran .

Nasal secretion from the sick calves were collected by using sterile cotton swabs. The swabs were immediately placed into screw capped bottles containing 1.5 ml. of ELY medium with antibiotics and were transported to laboratory in a thermos flask filled with ice cubes.

Specimen from the lung, tonsil, lymph nodes, tracheal and nasal mucosa of a dead calf were also collected and brought to the laboratory under the same condition.

The secretion absorbed to the cotton swabs was extracted into the ELY medium by pressing them with a pair of pincers. The extracts were then centrifuged at 10,000 r. p. m. for 20 minutes and the supernatant fluid was kept at -70°C. until being used as inoculum for virus isolation. Similarly, the extracts from tissue specimens was prepared from a 10 per cent suspension of each tissue specimen in ELY by using tissue grinder, and was kept at -70°C. until being used for virus isolation.

Cell cultures. primary monolayer of bovine foetal kidney (BFK) and lamb kidney (LK) cell cultures were prepared by the trypsinization method of Dulbecco and Vogt as modified by Younger (1959). Cells were grown in ELY medium containing 10 per cent inactivated calf serum, 100 units of penicillin, and 100 ug. of streptomycine/ml.

Secondary cultures of cells were prepared by adding a 0.25 per cent solution of trypsin to monolayers of cell cultures and growing the obtained cell suspension in the growth medium.

Virus isolation - Isolation of Virus was performed by inoculation of primary or secondary cultures of (BFK) cells with the above prepared inoculums. 0.1 ml. of each inoculum was inoculated into each of 4 cell cultures tubes. Cells were examined microscopically every day for the appearance of cytopathic effect (CPE). Further passages were also made in (BFK) cell cultures .

Antiserum preparation. - Attempts were made to prepare antiserum against the isolated strain in rabbits. The animals received 15 intravenous injection of 10 ml. of the virus suspension in its 3rd. passages in (BFK) cells. Sera were collected before the 10th. inoculation and 10 days after the last inoculation. The sera were kept at - 20°C. until required.

Neutralization test. - An 0.3 ml. of each of serial 10 fold dilutions of the virus was mixed with an equal volume of inactivated serum. For control, normal serum from the same species was mixed with the virus dilutions. The virus serum mixtures were incubated at -37°C. for 2 hours, and then tested for virus infectivity by inoculating 2 cell culture tubes per mixture. The extent of neutralization was expressed as neutralization log index (NI), which represented the difference between the titre of the virus in the presence of normal control serum and the titre of the virus in the presence of the serum to be tested.

Haemagglutination and haemadsorption tests. Tests were done in the usual manner using guinea pig erythrocytes.

Ether and chloroform sensitivity tests. - The sensitivity of the isolated virus to

ether and chloroform was tested by the method of Andrewes (1949), Feldman and Wang (1961) as previously described (Hazrati et al 1974).

Two bovine herpesvirus type 1 strains, Esfahan and American strain (Hazrati and Amjadi 1974), and strain WBRI of bovine adenovirus (Darbyshire et al 1966) were simultaneously used in the test as ether sensitive and ether resistance viruses, respectively.

Cytopathological studies. Cells grown on coverslips in Leighton tubes were infected and then, at appropriate time intervals, the cells were fixed with Carnoy fixative and stained with Harris haematoxylin eosin stains before being studied microscopically for cytological changes.

Electron microscopic study. - Virus was harvested from the infected BFK cell cultures showing about 90 per cent CPE. The infected cells were subjected to one cycle of freezing and thawing then centrifuged at 10,000 r.p.m. for 20 minutes . The supernatant was saved and used for electron microscopy.

A sample of the virus suspension was mixed with an equal volume of 3% phosphotungstic acid (PTA) PH 7.2 on formvar coated copper grids and examined in a philips EM 400 electron microscope.

Experimental transmission. - Four 1 to 3 month - old calves were used in exposure trial. The calves were selected from farms having no problem with respiratory disease and they were apparently healthy at the time of experiment.

Two calves (Nos. 1 and 3) were inoculated each intranasally (2.5 ml. of the virus fluid per nostril), conjunctively (0.5 ml. of the fluid per eye), and intratracheally (2.5 ml. of the virus fluid).

The inoculum was the fluid harvested from BFK cell culture infected with the virus isolate at its 3rd. passage level.

The other 2 calves (Nos. 2, and 4) were housed with the inoculated calves as contact controls.

The animals were examined daily and body temperature, heart beats, and breathing rates were recorded during the experiment.

Nasal secretions were collected for virus isolation during the observation period. Virus isolation was attempted also from different tissues of sacrificed calves. Pre-and post exposure sera were also collected to test the antibody response of the animals.

RESULTS

Clinical feature of the outbreak. - There were close similarities between the appearance and clinical aspects of the outbreak reported in this communication and those observed during the past 7 years in several other farms in Tehran.

The disease appeared among a group of 80 calves 1 to 4 month-old of a dairy farm consisting of about 1000 animals.

The first symptoms noticed were harsh hacking cough, when calves were forced to run. Affected animals showed a rise in temperature (up to 41.5°C.) at the onset of illness. With advancement of the disease coughing and panting became more frequent but the temperature, unless secondary infection occurred, usually became normal. In several cases coughing was associated with some mucoid discharge from the nostrils, animals stood apart and showed marked respiratory effect. Respiratory rate and heart beats increased accordingly, 70/min. and 120/min., respectively.

Mortality hardly exceeded 40 per cent at this stage but most of the affected calves went to a prolonged chronic form of infection and respiratory symptoms became more severe. In the majority of sick animals the nasal discharge, due to secondary bacterial infection, became mucopurulent and some calves showed a mild or severe signs of enteritis. There was, however, considerable loss in the condition of animals at this stage of the disease (Fig. 1) and the affected animals were slaughtered for economical reasons.

Post mortem examination on calves died or killed in acute stage of the disease revealed that the characteristic lesions were usually confined to the thoracic cavity and often consisted of lung consolidation only. The apical and cardiac lobes were most frequently involved but in some more advanced cases even more than two third of total lung tissues were affected. The affected tissue had a characteristic purple colour and were very firm in consistency.

In chronic forms of the disease, in which secondary bacterial infection occurred, abscess formation in lung tissue, pleural adhesion and even enteritis were noticed.

Virus isolation. - Three to five days after inoculation of BFK cell cultures with lung; lymph nodes, and tonsil tissues from 2 cases of dead and killed calves and with nasal swabs of a sick animal, a cytopathic changes were observed which were more evident in subsequent passages. The isolated agents were soon found to be very similar to each other in that they readily grew in BFK and LK cells producing indistinguishable CPE. Therefore one of the isolated strains, subsequently designated Razi 492, was chosen for further studies.

Properties of the virus. - Third to fifth passages of the virus strain in BFK cells were used to study the properties of the virus.

The virus produced a cytopathic effect (CPE) within 2 to 4 days of inoculation on BFK and LK cells. The first noticeable changes consisted of rounding and shrinking of infected cells. The degenerating cells were scattered throughout the cell sheet and produced no distinct foci of CPE. The infection spread gradually and affected the entire cell sheet resulting in the complete destruction of

the cell monolayers, within 5 to 10 days after inoculation, (Fig. 2). Acidophilic Cowdry type A intranuclear inclusion were observed in a large proportions of the infected cells but the virus did not produce characteristic syncytia in BFK cells under the condition of experiment (Fig. 2).

The virus completely lost its infectivity by ether and chloro form treatment. The ether sensitive controls, the strains of IBR virus, were also completely inactivated, whereas the ether resistant control, the adenovirus WBRL strain, was not inactivated.

The virus did not agglutinate erythrocytes of guinea pig and no haemadsorption of guinea pig erythrocytes was observed on cells infected with the virus.

Strain Razi 492 had the typical features of herpes virus group in negatively stained preparation. «Empty» and «Full» particles each with and without «Envelope» were present in the preparation. Naked virus particles were hexagonal and their surface, similar to other herpes viruses, was covered with hollow capsomers and had a diameter of 100 to 110 nm. The size of enveloped particles, due to the irregular shape of the envelopes, was difficult to determine, but was estimated to be approximately 170 to 350 nm, (Fig. 3).

Pathogenicity and Immunogenic properties of the virus. In an experimental transmission trial, the virus was found to be pathogenic to young calves. Two calves (Nos. 1 and 3) inoculated. Via the respiratory route, with the virus showed clinical signs of a typical respiratory infection very similar to those observed in naturally infected calves. The exposed animals developed respiratory illness accompanied by pyrexia, coughing, nasal discharge, hyperphnea, and dispnea. Temperature as high as 41.5°C., respiratory rate as high as 65 per minutes, and heart beat as much as 86 per minutes were noted on the day 6 in calf No. 1 (Fig. 4). This calf was sacrificed on day 11 for necropsy. Gross lesions were confined mainly to the respiratory system. The tracheobronchial pathways contained a whitish exudate, the mucous membrane was moderately hyperaemic and had numerous desquamated petechial haemorrhages. Areas of consolidation were observed in the apical, cardiac and anteroventral portions of diaphragmatic lobes of the lungs, (Fig. 5).

Histologically, engorgement of capillaries and thickening of alveolar walls, fibrinous deposition in the alveoli and intralobular septa were noticed. Necrosis of the tissues and abscessation were observed in different lobules. Most of the alveoli were heavily infiltrated with mononuclear, predominantly monocyte, cells, although a mild neutrophilic infiltration was also present in several areas.

The superficial epithelial cells of the tracheal mucosa desquamated falling down into the lumen. Eosinophilic intranuclear inclusion bodies were noted in some of the epithelial cells of tracheal and bronchial mucosa.

The clinical symptoms in calf No. 3 were not as severe as in calf No. 1. The infected calf resisted although respiratory disorders including coughing and signs

of consolidation in some parts of lung, remained up to the end of the experiment (for 30 days) .

None of the inoculated calves developed any sign of conjunctivitis.

The control calf No. 2 remained normal during the experiment. Calf. No. 4, suffering from enteritis was sacrificed on day 18. No significant Macroscopic changes were observed in the respiratory system . Histologically, slight submucosal infiltration was noted in traches and the alveolar walls were thickened by oedema and inflammatory cells.

Th virus was recovered in 2 occasion from nasal swabs collected from both inoculated calves and from the lung, lymph nodes, and tonsil tissue samples collected at necropsy of calf No. 1. The virus was also isolated from nasal swab collected from calf. No. 4, 15 days after being in contact with the inoculated calves. No virus was isolated from the organ tissue of this calf after being sacrificed.

Calves No. 2 and No. 3 were reinoculated with the virus 30 days after the beginning of the experiment. The animals were closely observed for 3 weeks without demonstrating any clinical symptoms. Those calves were scarificed and were examined for any pathological changes. Some small areas of consolidation was observed in lungs of both calves.

No neutralizing antibody was demonstrated in serum samples weekly collected from the inoculated calves.

The virus was found to be nonpathogenic to rabbits inoculated intravenously. Rabbits so inoculated produced only a low antibody response after 15 injections but antibody after 10 inoculations was not demonstrable.

Antigenic relationships. - The results of cross neutralization tests, table 1 indicated that the strain Razi 492 is antigenically distinct from bovine herpesvirus 1, bovine herpesvirus 2, and parainfluenza 3 viruses.

DISCUSSION

Outbreaks of respiratory infections in calves have been reported, as one of the most serious problems in certain countries where intensive feedlot or large dairy farms have been in practice. The outbreak may occur as acute clinical disease but usually observed as chronic forms. Heavy economical losses of the disease to cattle industries, however, are due to diminished body weight, high mortalities and expensive therapy. Various agents such as viruses, mycoplasma and bacteria have been postulated as the cause of the bovine respiratory diseases.

These agents either alone or in various combination have been isolated from cases of the natural outbreaks and it has been shown that simultaneous infection with these agents was the cause of most of the outbreaks (Omar 1966). In addition

to infectious agents physical stresses, specially in intensively reared calves, were shown to be also of special significance. Viruses, among other infectious agents, were shown to play a more important role in the respiratory diseases of calves. Bovine herpesvirus 1, parainfluenza 3 Virus, and certain types of adeno, entero, rhino and reoviruses have been specially shown to be capable of causing respiratory diseases in calves (Darbyshire et al 1966, Lamont et al 1968, Madin et al 1956, Reisinger et al 1959) During last decade several strains of herpesvirus, were isolated from calves suffering from respiratory disease, (Bartha et al 1966, Kryukov et al 1971). Some of these strains, namely DN- 599, FTC-2, ST, 66, were shown to be pathogenic for young calves, but some others, namely Movar 33/63, failed to elicit any clinical diseases in experimentally exposed calves (Bartha et al 1967). These virus strains, however, were classified as bovine herpesvirus 3 and were added to the viral agents with possible etiological role in calf respiratory disease complex (Gibbs and Rweyemamu 1977).

Isolation of a new strain of bovine herpesvirus 3, as described in this communication, from cases of pneumonia in calves could be considered as further evidence of the etiological importance of this group of viruses in bovine respiratory diseases. This was confirmed by showing that the virus was capable of causing respiratory infection in experimentally exposed calves. The infected calves developed clinical signs of pneumo - enteritis characterized by fever, coughing, dyspnea, nasal discharge and a mild diarrhea. In the necropsy of one of the inoculated calves, killed on day 11 after exposure, area of consolidation and emphysema in the lungs, congestion in turbinate and tracheal mucosa and enteritis were noticed. The virus was recovered in 2 occasions from nasal swabs collected from infected calves and from lung. Lymph nodes, and tonsil tissue samples at necropsy of the sacrificed calf.

Although several microorganisms were isolated from lung tissue of the sacrificed calf it is presumed that the virus was the primary etiological agent of the infection and was responsible for the pathological changes in the respiratory tract tissues. It appeared that the virus was very poor antibody producer in infected animals. Antibody was demonstrated neither in any of 25 calves in the farm from which the virus was isolated, nor in the calves experimentally inoculated once or twice with large doses of the virus. The poor antigenicity of the virus was also demonstrated by inability to detect specific neutralizing antibody in rabbits after 10 , weekly interval, intravenous inoculations of the virus . The antibody, however, was produced by 15 inoculations in this animal. The results, however, confirmed the findings of other workers on the extremely poor antigenicity of other herpesvirus 3 strains previously isolated from cases of calf pneumonia in other parts of the world (Mohanty 1973, Mohanty et al 1972).

It has been shown previously that calves recovered from infection with herpesvirus 3 strain DN-599 were immune when challenged 8 weeks after infec-

tion. A similar response was observed in present study by using strain Razi 492 indicating that the virus might have the potentiality of producing some sort of immunity in exposed calves.

The pathogenicity and immunogenicity of bovine herpesvirus 3, however, has not been well defined and still more critical work is needed to elucidate the role of this group of herpesvirus in respiratory disease, complex. Biological and morphological characteristics of the virus strains, on the other hand, has been studied in more detail (Batha et al 1967, Munz et al 1974, Liebermann et al 1967).

Strain Razi 492 grew well in bovine foetal kidney and lamb kidney cell cultures producing CPE within 2 to 4 days. Cowdry type A intranuclear inclusions were observed in a large proportion of the infected cells but the virus did not produce characteristic syncytium in BFK cells.

The virus had characteristic features of herpesvirus group in negatively stained preparations. Naked virus particles diameter was estimated to be 100 to 110 nm.

The virus was shown to be serologically distinct from bovine herpesvirus 1 and bovine herpesvirus 2, but have the general properties of herpesvirus group.

Table.; 1. Cross neutralization tests between strain Razi 492 and strains of bovine herpesvirus 1 (American st.), bovine herpesvirus 2 (st. 69/1 LO) and parainfluenza 3 (strain T.1) viruses.

Antiserum	Virus strains			
	Razi 492	American	69/ILO	T.1
Razi 492	3.5+	0	0	0
Bovine herpesvirus 1 (American st.)	0	5.5	0	0
Bovine herpesvirus 2 (st. 69/ILO)++	0	0	5.5	0
Parainfluenza 3 (st. TL1)	0	0	0	5.5

+ = Neutralization log-Index (NI).

++ = Provided through the courtesy of Dr. G. Castrucci (Istituto di Malattie Infettive, Profilassi e polizia Veterinaria della Università di Perugia, Italy).

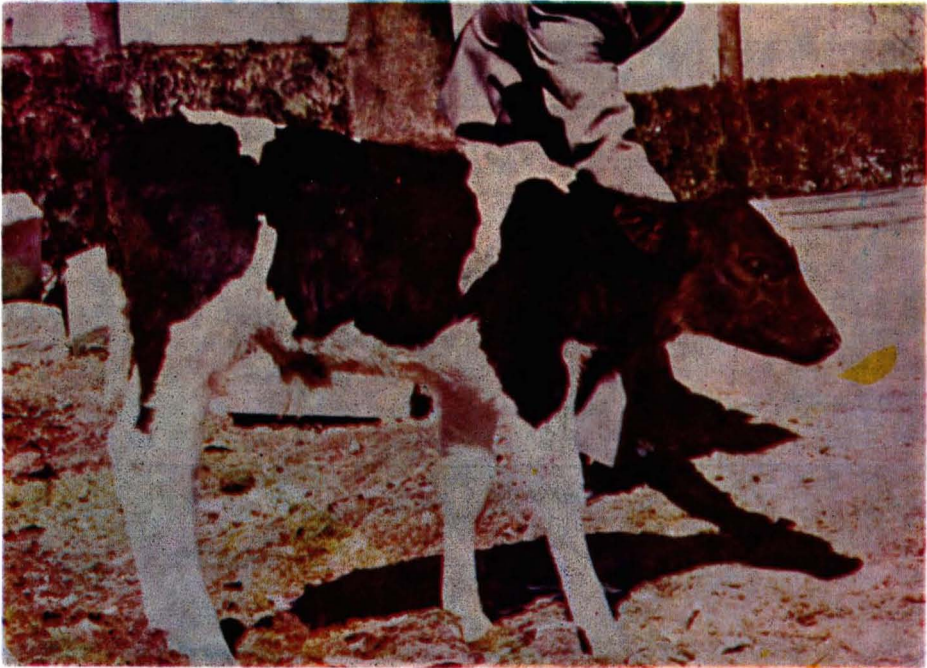
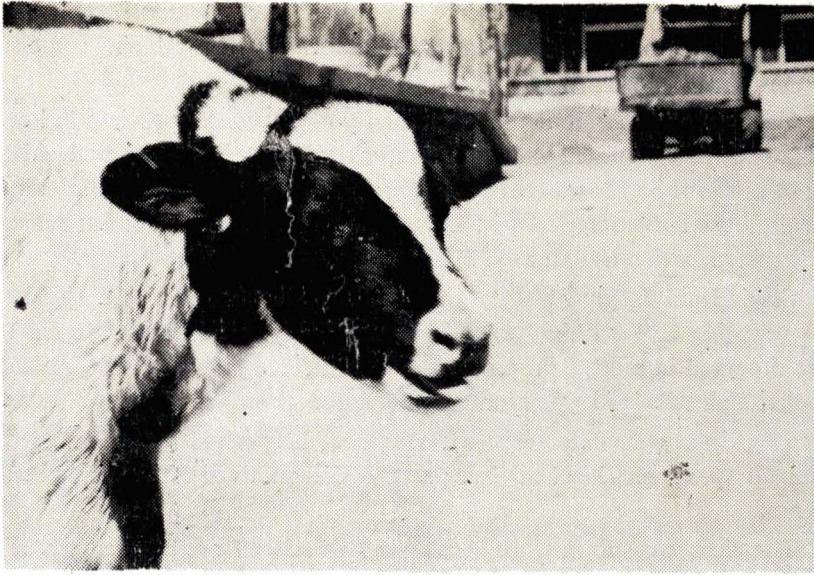


Fig. 1- Naturally infected calves showing marked respiratory disorders and considerable loss in condition.

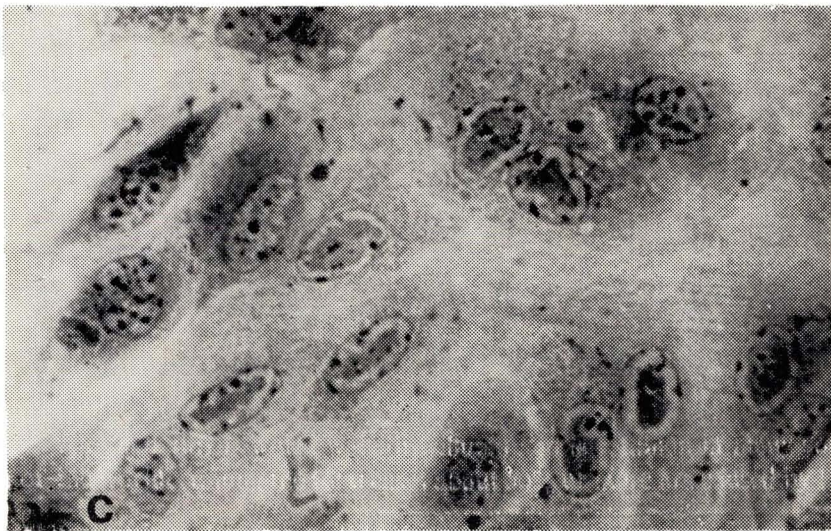
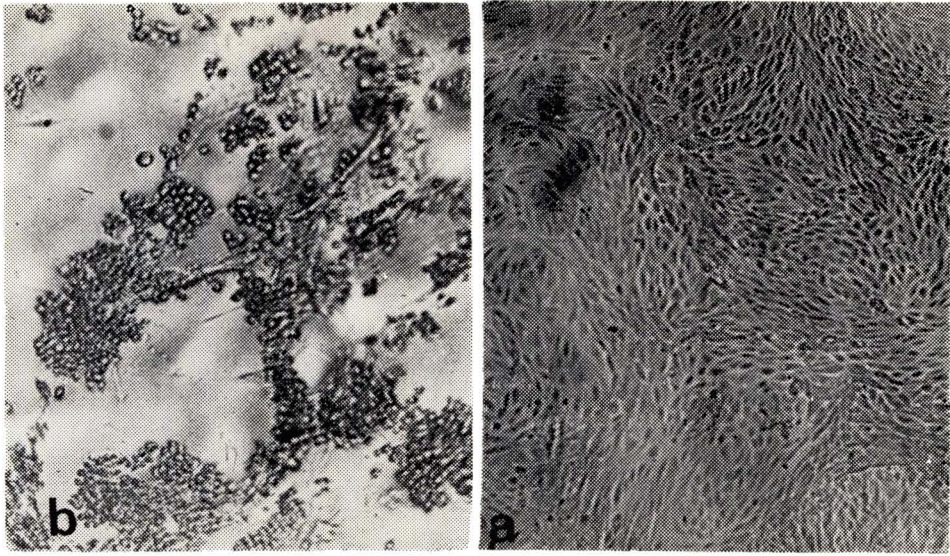


Fig. 2- Normal BFK cell cultures, (a), typical cytopathic effect, (b), and intranuclear inclusion (c) in cells infected with strain Razi 492 four days previously.

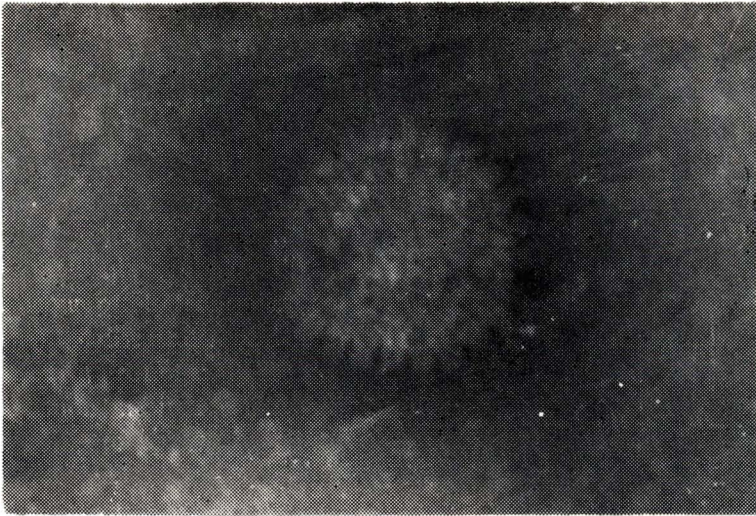
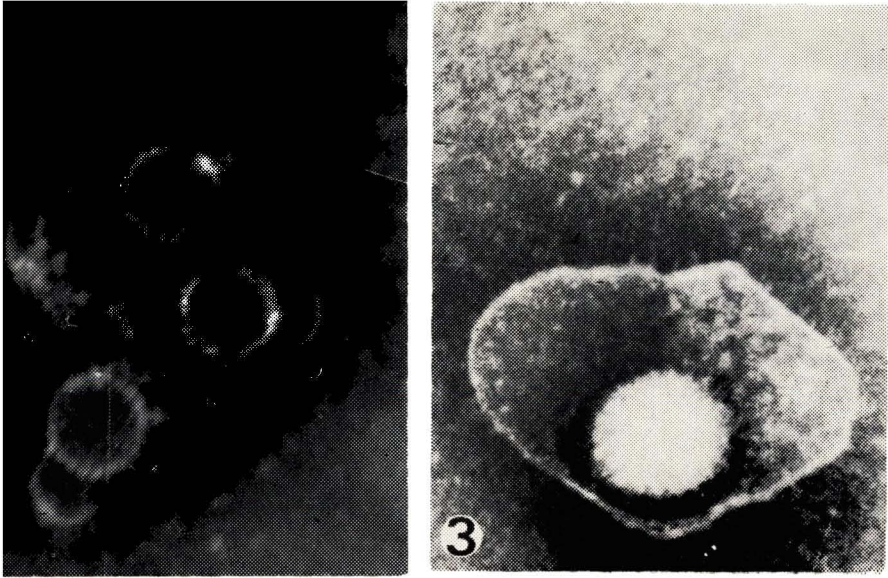


Fig. 3- Negatively stained preparation showing virus particles of Razi 492 strain.

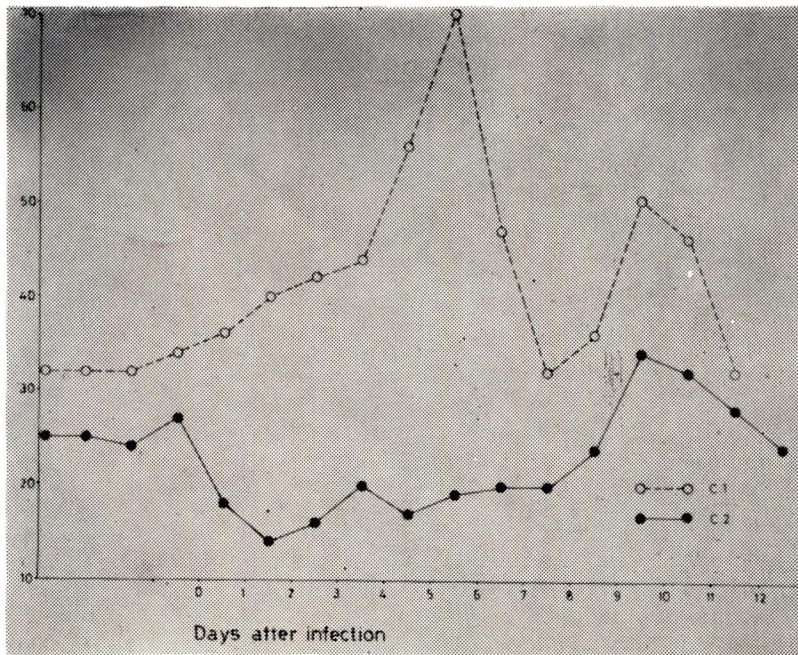
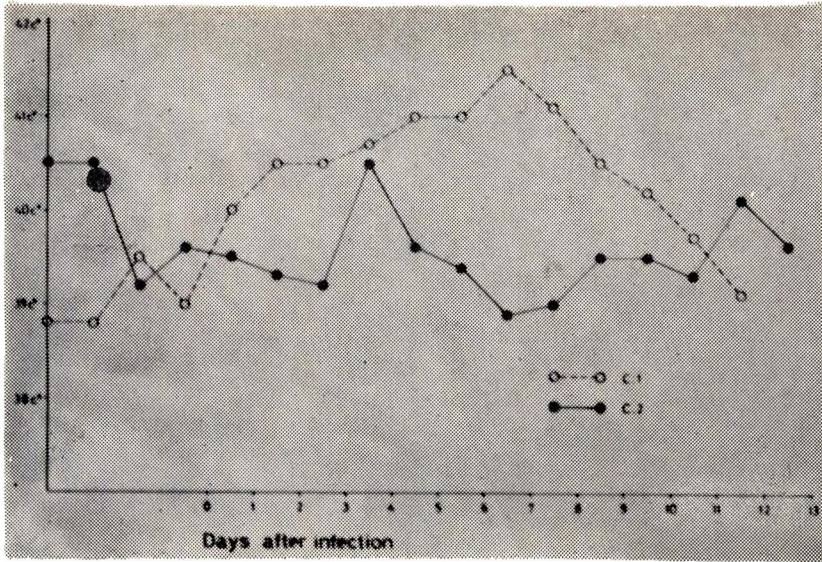
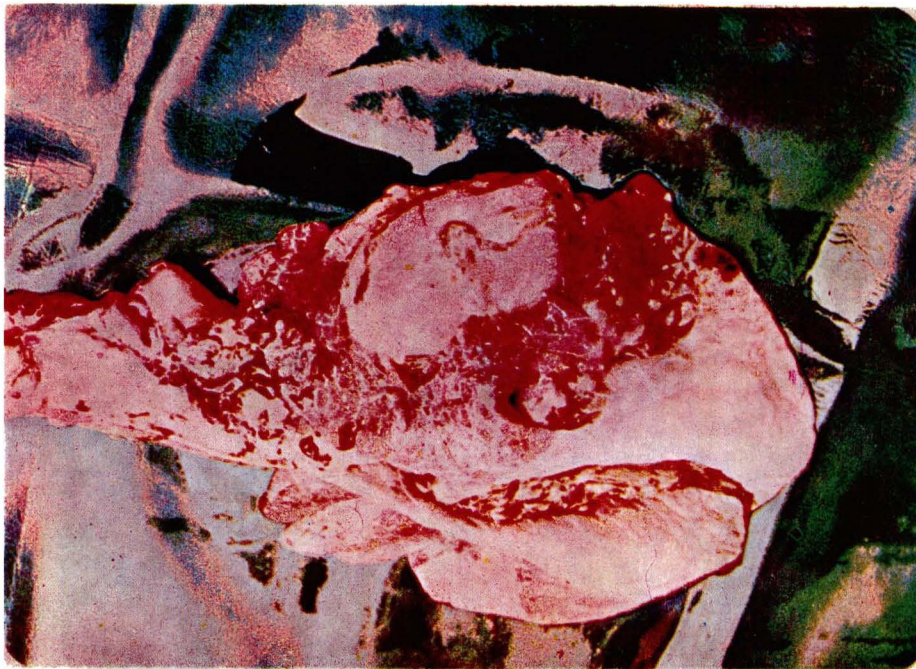
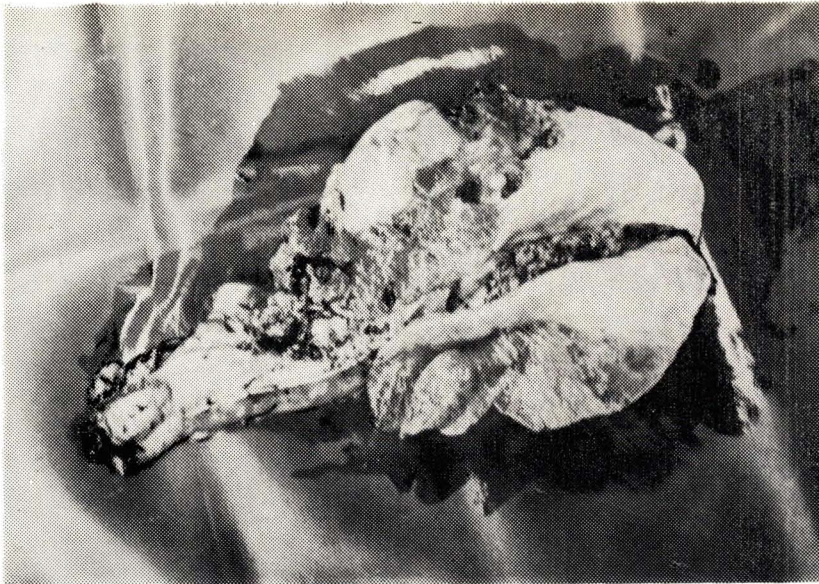


Fig. 4- Variation of body temperature and respiratory rates in calf No. 1, (c), inoculated with strain Razi 492 and in control calf No. 2, (c2).



F.g. 5- Lung of calf with area of consolidation II days after exposure to virus strain Razi 492.

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