Isolation of Bovine Ephemeral Fever Virus from 2004 outbreak in Minoufia Governorate

By

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Abstract

Isolation of BEF virus from leucocytic fractions of feverish cattle by blind intracerebral inoculation in baby mice was confirmed by positive Immunoperoxidase on fixed preparations of infected mice brain. Histopathological examination of infected mice brain showed the pathological changes of the brain tissue which explained the nervous manifestations observed on inoculated baby mice. BEF virus was also isolated on VERO cells which were inoculated by reconstituted suspension of infected mice brain. The isolation on tissue culture was confirmed by observation of specific cytopathic effect and by electron microscopy which showed the virus particles in the cytoplasm of infected VERO cells.

Introduction

The diagnosis of BEF depends on detection of virus antigen by immnufluorescence, and virus isolation and identification as well as detection of specific antibodies in paired serum samples by neutralization test (Tzipori, 1975).

Material and methods

Baby mice:

A total number of 232 baby mice (1-2 day old) were used for intracerebrally inoculation with the leucocytic fraction of feverish cattle or brain emulsion of previously inoculated mice for isolation of bovine ephemeral fever virus.

Blood and serum samples:

Heparinized blood samples were collected from cattle for separation of buffy coat for virus isolation.

Cell culture:

The following cell lines were used for isolation, propagation and titration of bovine ephemeral fever virus.

1) Baby hamster kidney cell line (BHK21) established by Macpherson and stocker (1962).

2) African green monkey kidney cell line (VERO) established by Yasummara and Kawatika (1963). They were kindly supplied by Serum and Vaccine Research Institute, Abasia, Cairo, Egypt.

Antibovine and antirabbit peroxidase conjugate:

Antibovine and antirabbit immunoglobulines conjugated with horse raddish peroxidase are commercially available (BETHYL LABORATORIES, INC.).

Separation of buffy coat:

It was carried out as follows:

- About 8 ml of heparinized blood samples were collected from animals during febrile phase in 15 ml test tube.
- Centrifugation at 1500 r.p.m for 20 minutes was followed.
- Discard the supernatant plasma layer.
- Aspiration of buffy coat layer by Pasteur pipette.
- The buffy coat was separated and washed three times by adding distilled water and centrifugation.
- Clear buffy coat sediment was suspended in distilled water and preserved in ependorf at –20°c (Nagano et al., 1990).

Isolation of BEF virus by intracerebral inoculation of baby mice:

The intracerebral inoculation of leucocytic fraction of feverish cattle or brain suspension of previously inoculated suckling mice (48-96 hours age) was done as follows:

The first passage was done by injection of 0.01ml of 10% leucocytic fraction of feverish cattle in normal saline .Passages from second to seventh were done by injection of 0.01ml of 10% mice brain suspension in normal saline. Baby mice were kept under observation until nervous symptoms appeared or slaughtered at 21 days old.

Isolation of BEF virus in cell culture:

A confleunt monolayer of BHK 21 and VERO cell cultures 50 ml prescriptions were inoculated with o.2ml/prescription of 10% leucocytic fraction or previously inoculated mice brain in normal saline and left for one hour to allow virus adsorption. Subsequently infected cells were washed with HBSS then

supplemented with maintenance media and incubated at 37°c and subjected to daily microscopical examination to detect the induced cytopathic effects.

Histopathology of mice brain:

Infected mice brain was prepared for histopathological examination according to Bancroft and Gamble (2002)

Immunoperoxidase technique:

It was carried as described by Zaghawa (1989).

Electron microscopy for cell culture infected with BEF virus:

Electron microscopy was carried for cell culture infected with BEFV isolate according to Kay, (1965)

Results

Isolation of BEF virus in mice after intracerebral inoculation of leucocytic fraction from naturally occurring cases during the summer outbreak 2004:

Clinical observation of inoculated mice:

Table (1) showed different clinical manifestations of mice inoculated intracerebrally with leucocytic fraction from cattle clinically affected with bovine ephemeral fever virus. The clinical signs appeared firstly with arched back, circling, paresis, paralysis and finally death. The severity of clinical signs is different according to the level of passage and isolate.

Table (1): Clinical observation of baby mice inoculated with buffy coat from clinically affected cattle with bovine ephemeral fever virus.

Passage NO.	Clinical observation
First	Death of some inoculated mice over 8-12 day post inoculation. Apparently healthy mice were killed 21 day post inoculation.
Second	Some inoculated mice suffered from arched back other inoculated mice appear normal till killed 21 days post inoculation.
Third	Some inoculated mice suffered from arched back other inoculated mice appear normal till killed 21 days post inoculation.
Fourth	Some inoculated mice suffered from arched back other inoculated mice appear normal till killing 21 days post inoculation. Fig. (10).
Fifth	Tremors, arched back, paresis of hind quarter and death in some inoculated mice 8-12 days post inoculation. Fig. (11).
Sixth	Tremors, arched back, paralysis of hind quarter and death in some inoculated mice 6-10 days post inoculation. Fig. (12).
Seventh	Tremors, arched back, paralysis of hind quarter and death in some inoculated mice 2-5 days post inoculation. Fig. (13).

Immunoperoxidase of brain smears:

Immunoperoxidase (IP) technique was applied on inoculated mice brain smears and showed (fig.1) intense reddish brown staining intracytoplasmic granules in brain cells indicating a positive reaction.

Histopathology of inoculated baby mice brain:

Histopathological changes noticed in the first passage were in the form of hyperemia in cerebral and menegial blood vessels fig. (2), perivascular oedema, neural picnosis and demylination.The changes in the second passage included perivascular hemorrhage and oedema in addition to neural picnosis and demylination fig. (3). the third and fourth passage showed the same changes in the second passage together with focal early malacia fig. (4).

In the fifth passage vascular phenomena (hyperemia, hemorrhage and oedema) subsides while gliosis and neural picnosis became oblivious fig. (5).

The sixth passage revealed diffuse neural picnosis, demylination and malacia fig. (6).

Malacia was the characteristic feature in the seventh passage fig. (7).

Isolation of BEF virus in VERO cell cultures:

Cytopathic effect (CPE) of bovine ephemeral fever virus:

Daily observation of inoculated VERO cell culture revealed appearance of CPE as cell rounding, granulated cytoplasm, cell aggregation and cell lysis that end with detatachment of cells from the culture surface 4 days in the first and second passages, after 2 days post inoculation in the third passage and 24 hours post inoculation in the fourth and fifth passages fig. (8).

The intensity of CPE was the latest in the first passage and increased in the second passage reaching its peak in the third passage and decrease again by the fourth and fifth passages.

Detection of bovine ephemeral fever virus particles in infected VERO cells by Electron Microscopy (E.M):

The detection of bovine ephemeral fever virus particles in infected VERO cells by Electron Microscope (E.M) was shown in fig. (9). Viral particles were observed within the cytoplasm of VERO cells and appeared as cone shaped, and sometimes circular when cutted out.

Discussion

The result of clinical manifestation of mice after intracerebral inoculation with leucocytic fraction from cattle clinically affected with bovine ephemeral fever virus as shown in table (١). It is clear from the table that the graduation of the nervous manifestation beginning from arched back, circling, paresis, paralysis and finally death. The severity of clinical signs is different according to the level of passage and isolate.

Theses results are in accordance with St.George et al., (1977) who stated that the virulent strains become estabilized after the six passage causing paralysis and death 2-3 days post inoculation. At the regional level Hassan et al. (1991) and Nawal et al. (2001) reported tremors, convulsions, and paralysis of mice at the third passage. Nagi et al. (1992) reported neurological signs manifested by tremors, convulsions and final paralysis were seen after the 3rd blind passage.

The histopathological findings of the inoculated mice brain were clear in figures (2, 3,4,5,6 and 7). The histopathological changes began with hyperemia, oedema, picnosis and end with demylination.

It is clear that the progress of these changes correlated with the passage level. Theses results are in accordance with Van der westhuizen (1967) after intracerebral mice inoculation and also with Inaba et al., (1968) after intracerebral inoculation of suckling hamster and rats.

Confirmation of the diagnosis by the immunoperoxidase staining of mice brain smears revealed intracytoplasmic intense reddish brown denoting the virus antigen in brain cells. These results agree with Khalil et al. (2001) and indicate that immunoperoxidase staining test is simple rapid and sensitive test for detecting bovine ephemeral fever virus antigen in brain smears or formaline embedded sections from other organs as well as in infected VERO cells. Fig (1). These results are supported by St. Georg (1988), Hassan et al., (1991) and Nawal et al., (2001). It is clear that effects were inhibited by incubation of the isolate with specific hyper immune serum to BEF virus.

The detection of bovine ephemeral fever virus particles in infected VERO cells by Electron Microscope (E.M) was shown in fig. (10). Viral particles was observed within the cytoplasm of VERO cells and appeared as cone shaped,

sometimes circular when cutted out. The electron microscopy is away for confirmation of BEF diagnosis as described by Chiu and Liu (1984) in Taiwan describing the general morphology of rhabdovirus under E.M. At the local regional situation in Egypt described the bullet-shape of BEF virus in infected VERO cells is a good diagnostic tool for confirmation of BEF viral infection in Egypt during summer 2004.

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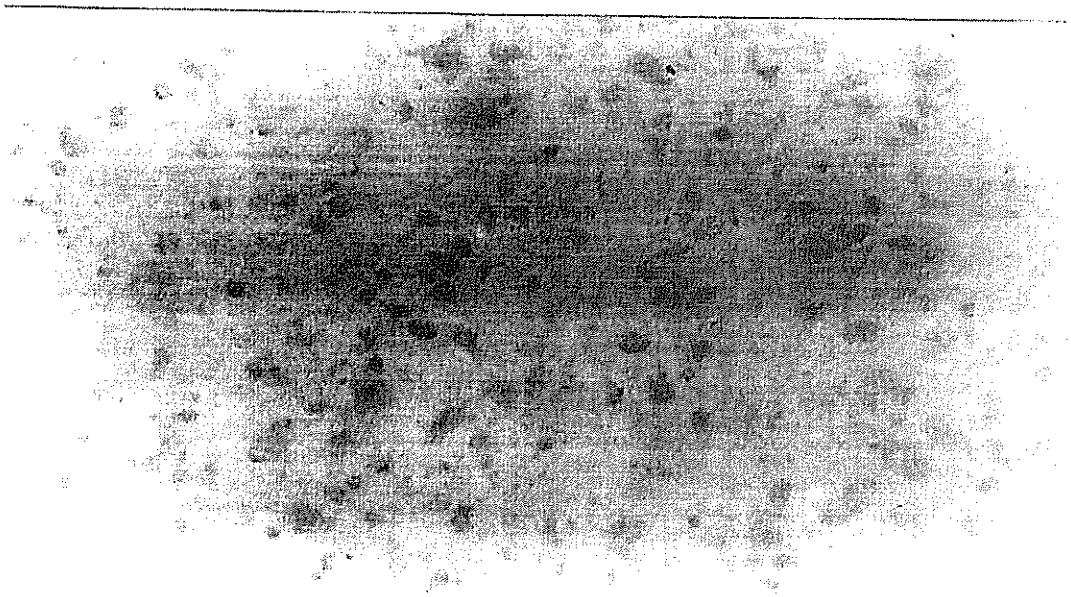

Fig. (1): Positive immunoperoxidase showing reddish brown staining intracytoplasmic granules in baby mice brain.

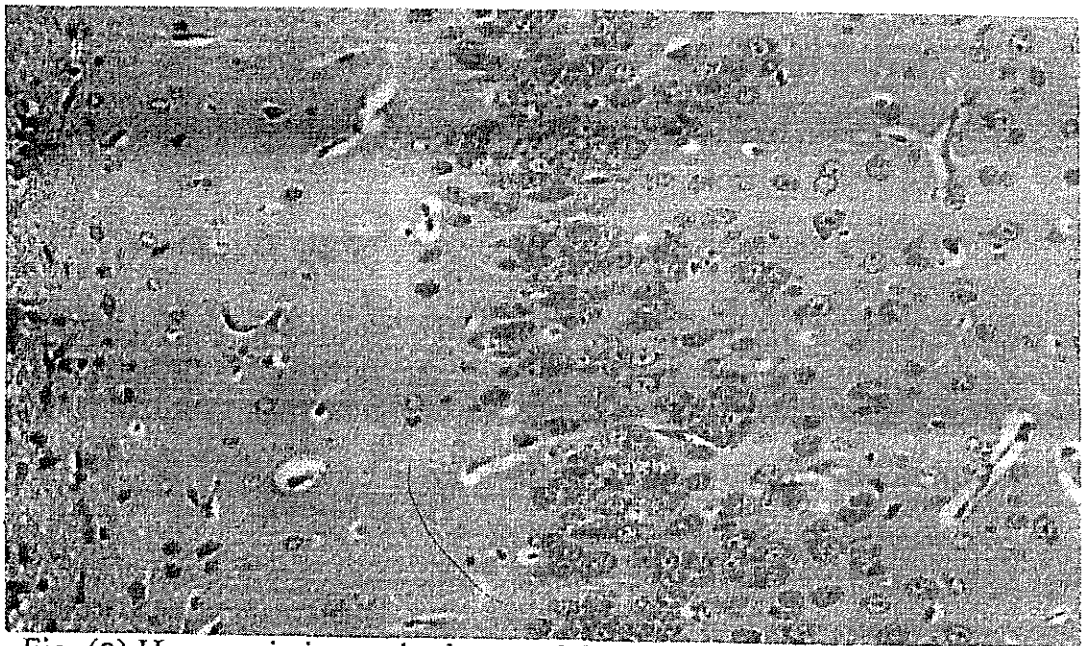

Fig. (2) Hyperemia in cerebral menegial blood vessels of baby mice brain inoculated with BEF virus passage No 1 (H&E stain, magnification: X20).

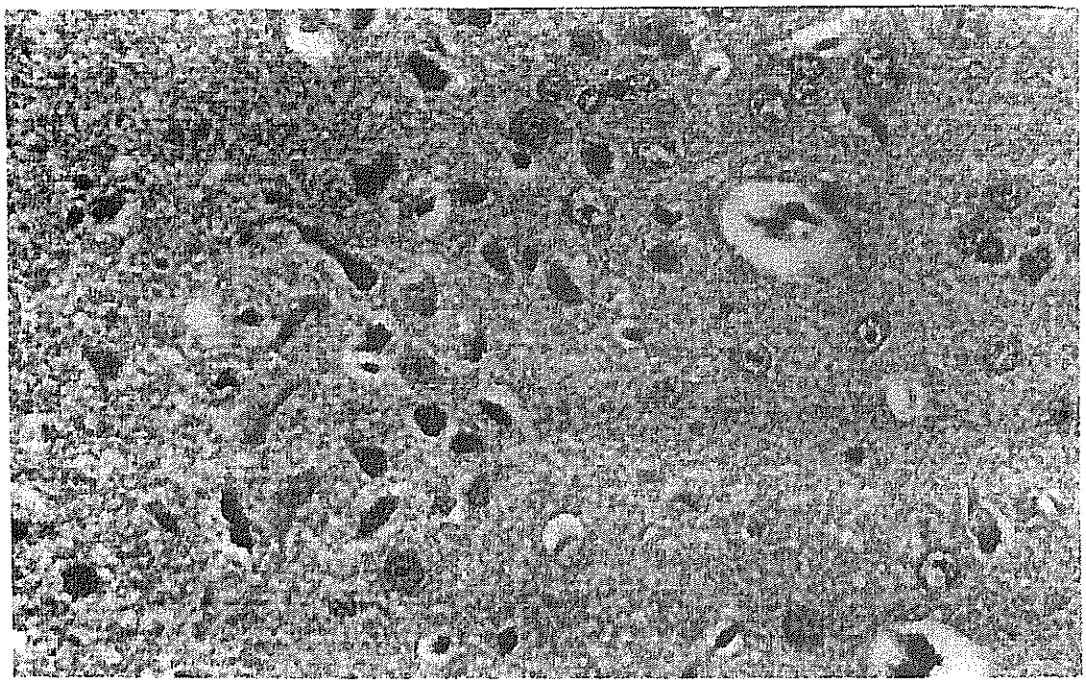

Fig. (3) Neural picnosis in cerebrum of baby mice brain inoculated with BEF virus passage No 2 (H&E stain, magnification: X20).

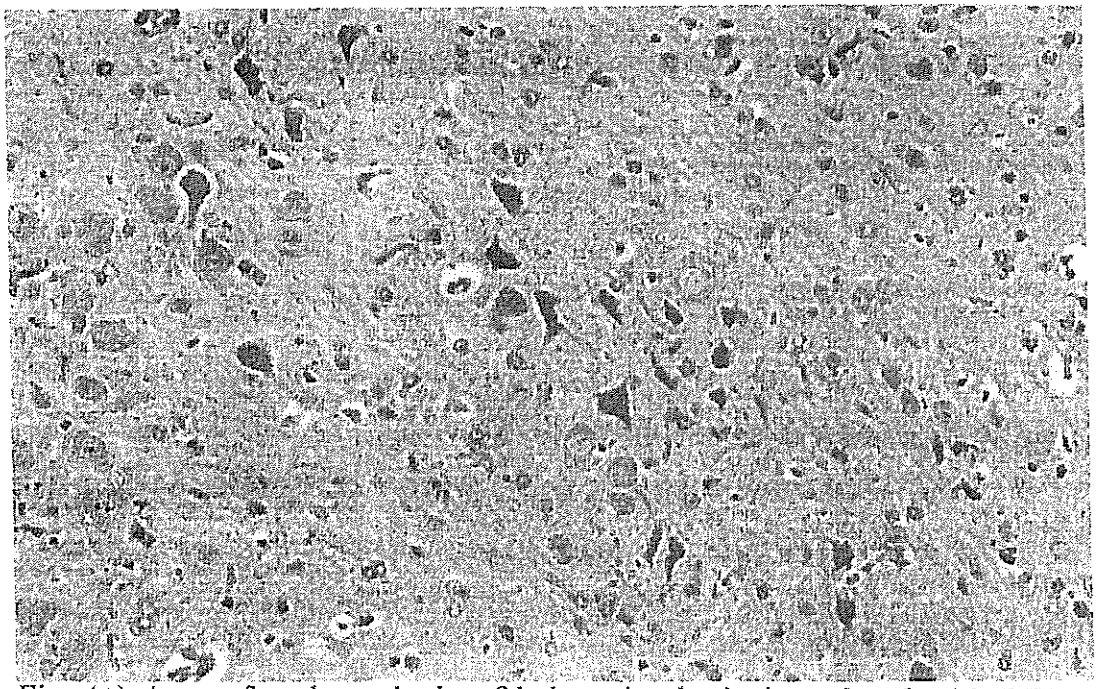

Fig. (4) Area of early malacia of baby mice brain inoculated with BEF virus passage No 3 (H&E stain, magnification: X40).

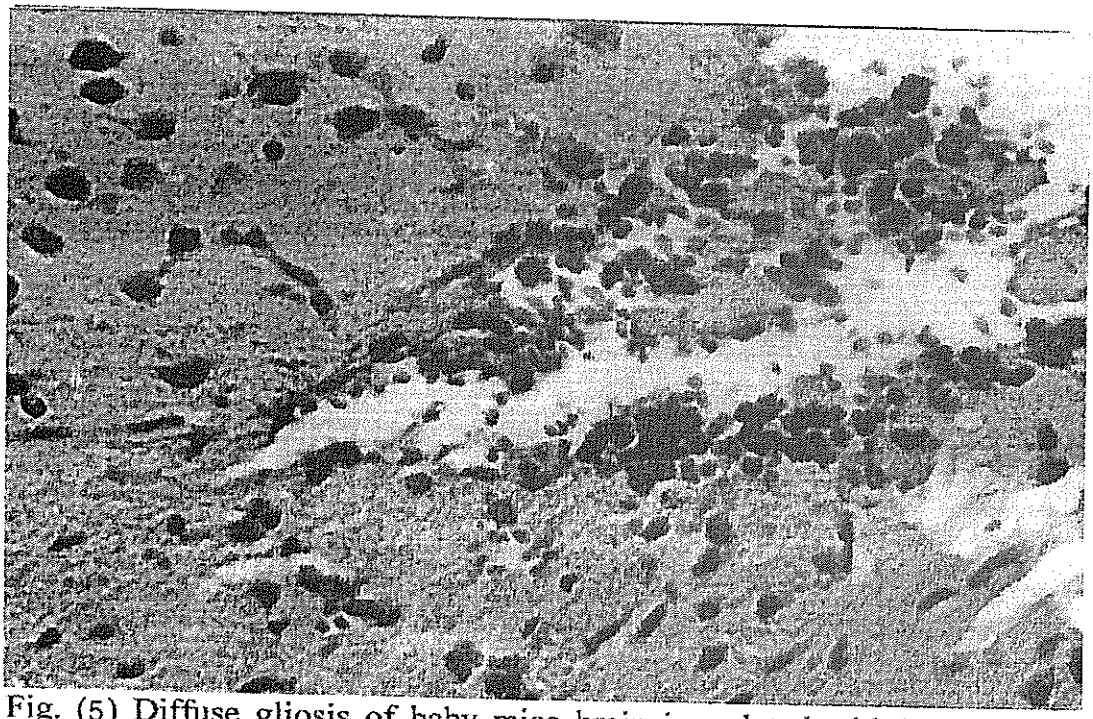

Fig. (5) Diffuse gliosis of baby mice brain inoculated with BEF virus passage No 5 (H&E stain, magnification: X40).

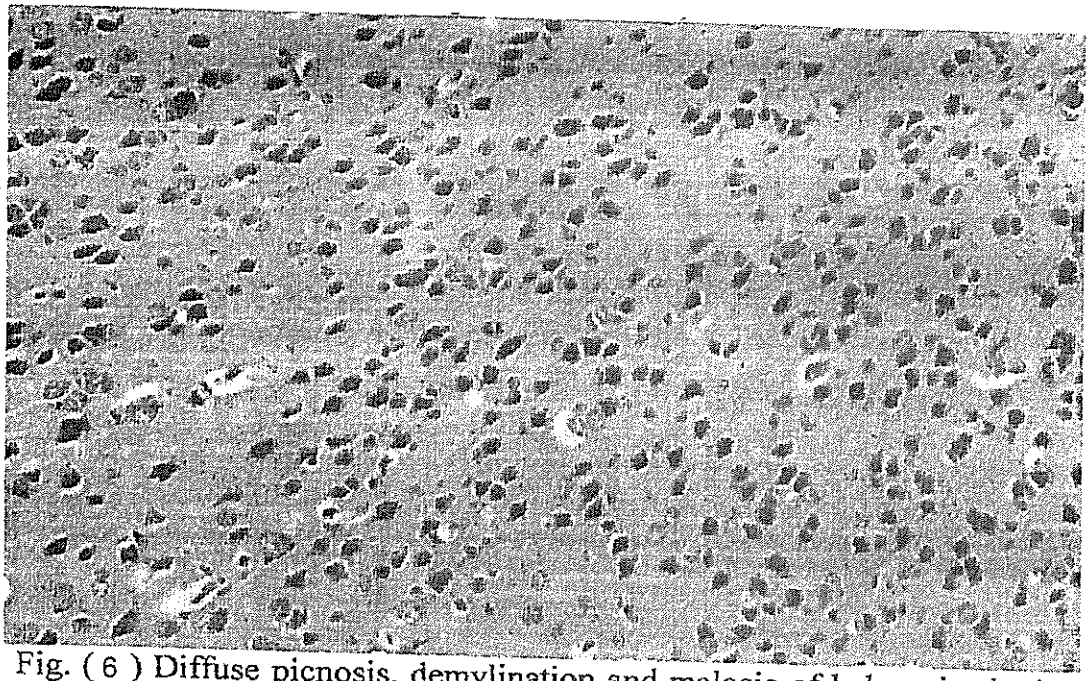

Fig. (6) Diffuse picnosis, demylination and malacia of baby mice brain inoculated with BEF virus passage No 6 (H&E stain, magnification: X40)..

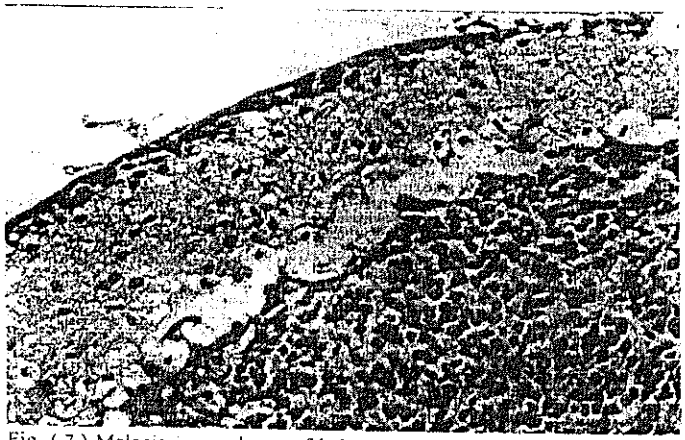

Fig. (7) Malacia in cerebrum of baby mice brain inoculated with BEF virus passage No 7 (H&E stain, magnification: X40).

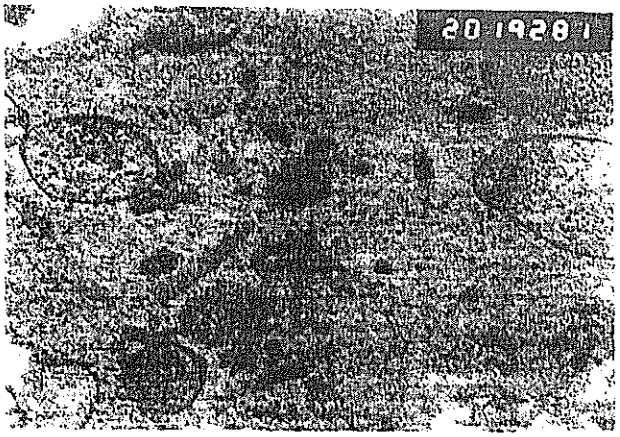

Fig. (9) BEF virus particles in the cytoplasm of infected VERO cells by electron microscope (magnification: X 20,000).

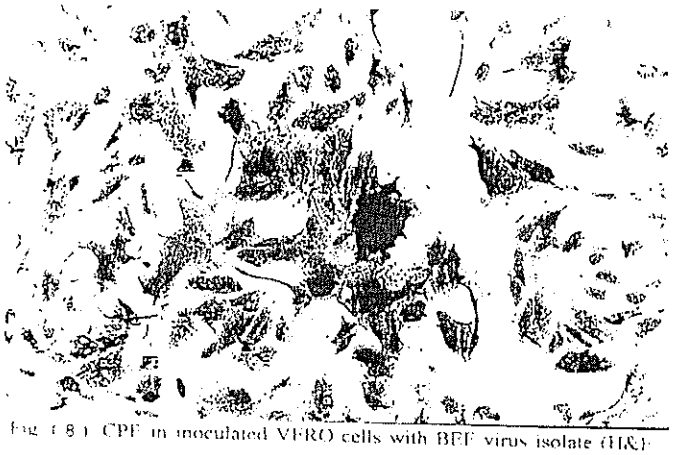

Fig. (8) CPE in inoculated VERO cells with BEF virus isolate (H&E stain, magnification X40)

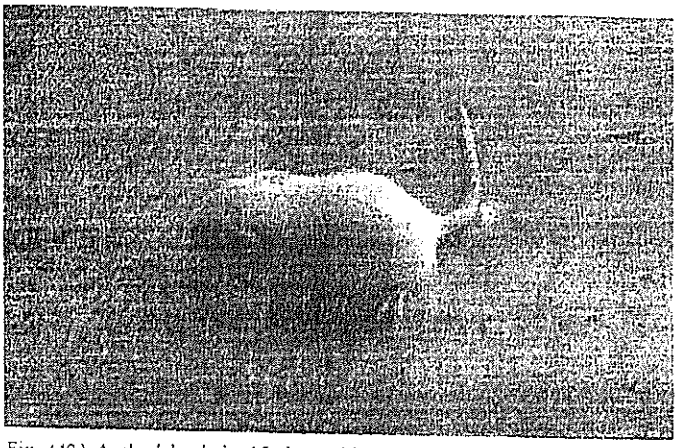

Fig. (10) Arched back in 15 days old baby mice inoculated with BEF virus isolate passage 4

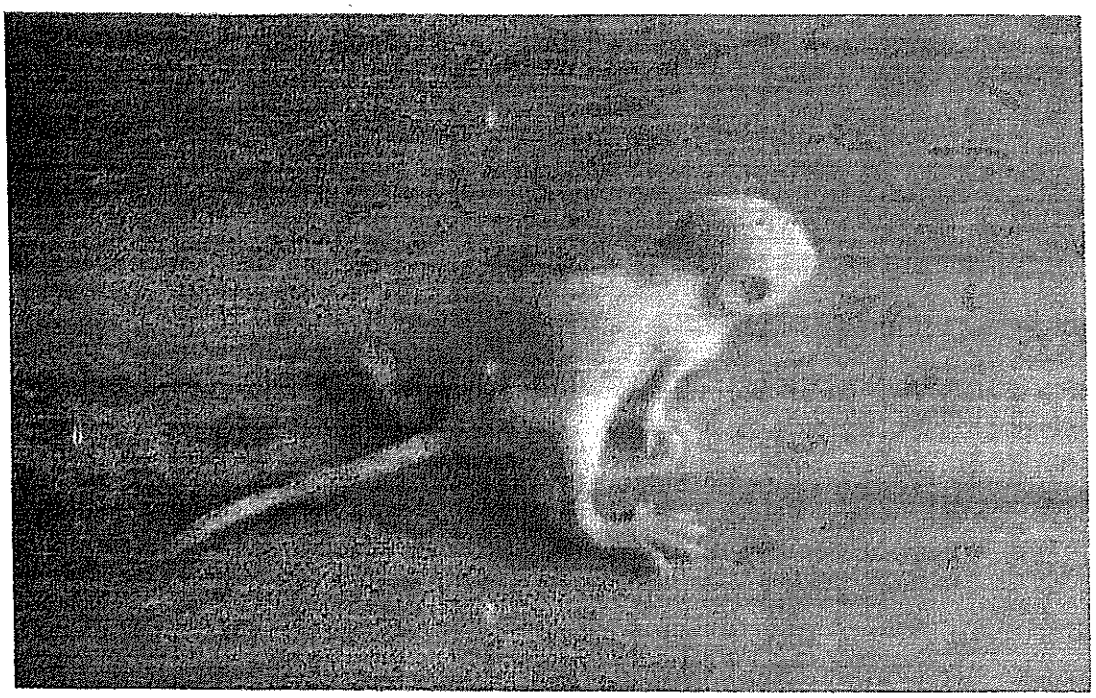

Fig. (11) Paresis of both hind legs of 12 days old baby mice inoculated with BEF virus isolate passage 5.

Fig. (12) Paralysis of both hind legs and tail of 10 days old baby mice inoculated with BEF virus isolate passage 6.

Fig. (13) Paralysis of both hind legs and tail of 5 days old baby mice inoculated with BEF virus isolate passage 7.

الملخص العربى

تم عزل فيروس حمى الثلاثة أيام بالتمرير فى مخ رضيع الجرذان وقد ظهرت أعراض عصبية على الجرذان المحقونة وتم تأكيد العزل من خلال عمل الأمينوبيروكسيداز على مسحات من مخ الجرذان المحقونة.

الخاص CPE فيرو وتم تأكيد العزل من خلال ملاحظة وقد تم العزل أيضا على خلايا بالفيروس وتثبيطها بالمصل المتخصص لفيروس حمى الثلاثة أيام.

تم التعرف على فيروس حمى الثلاثة في خلايا الفيروس باستخدام الميكروسكوب الإلكتروني والتي تتميز بشكل طلقة البندقية.Rhabdoviruses عن طريق الشكل الظاهري لمجموعة