RETROSPECTIVE; CLINICAL; LABORATORY AND ELECTRON MICROSCOPIC STUDIES ON BOVINE EPHEMERAL FEVER IN GOVERNMENTAL FARM IN MENOUFIA

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SUMMARY

Bovine ephemeral fever is an arthropod-borne rahbdovirus, which causes disablang febrile infection of cattle and water buffalo. The objectives of these studies were planned to analyze 3 Egyptian outbreaks occurred during 2000, 2001 and 2004 epidemiologically and clinically with special references to virological, molecular diagnosis and electron microscopic investigation.

The results indicated sever clinical disease with great economic losses during outbreak of summer 2000. The percentage of clinical disease was 49.87 % and 31.87 % in dairy and beef cattle respectively. The mortality rates were 2.66 % and 2.19 % in dairy and beef cattle respectively. The second outbreak occurred during summer 2001 was very mild in which the percentage of clinical disease were 18.8 % and 12.67 % in dairy and beef cattle respectively. The mortality rates were 0.7 % and 0.8 % in dairy and beef cattle respectively. The third outbreak occurred during summer 2004 was moderate one with percentage of clinical disease of 35.6 % and 36 % in dairy and beef cattle respectively. The mortality rates were 1.5 % and 0.9 % in dairy and beef cattle respectively.

The BEF virus was isolated by intracerebral inoculation of suckling mice and VERO cell culture. BEF virus was identified by immunoperoxidase staining. The positive reaction was seen as intense brown intracytoplasmic color. Viral particles were observed within the cytoplasm of endothelial cells and appeared as cone-shaped. The BEF virus genome was identified by reverse transcriptase polymerase chain reaction (RT-PCR). The running PCR products on gel electrophoreses gave a single band (500 base pair in length) indicating positive results. In conclusion, the experience trials to control the disease by vaccination with both killed and live attenuated vaccines failed.

The retrospective analysis of the data concluded that the level of herd immunity resulted from natural infection and the virulence of the BEF virus affect the severity of the outbreak. Further studies are needed to produce commercially a genetically engineered vaccine for control of the disease.

Kay words: Bovine ephemeral fever, epidemiology, PCR, Immunoperoxidase and BEF virus isolation.

INTRODUCTION

Bovine ephemeral fever (BEF) is a disabling viral disease of cattle and water buffaloes. It can cause significant economic impact through reduced milk production in dairy herds, loss of condition in beef cattle and loss of draught animals at the time of harvest. Available evidence indicates clinical signs of BEF, which include bi-phasic fever, anorexia, muscle stiffness, ocular and nasal discharge, ruminal stasis and recumbency, are due primarily to a vascular inflammatory response (Walker, 2005).

During summer 2000, a severe outbreak of BEF was recorded in Egypt. Clinically the disease occurred suddenly in both foreign and native breeds of cattle with severe economic losses (Zaghawa et al., 2000). Hematological and biochemical changes of the diseased animals in the same outbreak were described by Hassan (2000). This was followed by a mild outbreak during summer 2001 (El-Nagar, 2003). A relatively sever outbreak was observed during summer 2004.

The diagnosis of BEF depends on detection of virus antigen by immunofluorescence, and virus isolation and identification as well as detection of specific antibodies in paired serum samples by neutralization test (Tzipori, 1975). The application of immunoperoxidase technique for diagnosis of BEF virus infection was carried out by Khalil et al, (2001) as they describes the positive results as intense reddish brown staining intracytoplasmic granules in leucocytes specially neutrophils. They concluded that the test is simple, sensitive and can be considered as field test in the presence of the diagnostic reagents. Reverse transcriptase polymerase chain reaction (RT-PCR) has been developed with many advantages as it is possible to detect as little as 2 fragment of viral RNA from infected tissues by ethedium bromide staining after 30 cycle of PCR (Wu et al.,1992). There is no need for virus replication, moreover RT-PCR is not time consuming since all procedures involved take about 6 hours to be completed (Davis and Boyle, 1990). At the regional level in Egypt the RT-PCR is applied for BEF virus diagnosis by Khalil et al., (2001).

The aim of the present work was planned to fulfill the followings:

1) Clinical description and analysis of the last 3 outbreaks (2000, 2001 & 2004) occurred in a governmental farm at Menoufia province.

2) Isolation of BEF virus in suckling mice and VERO & BHK cell lines followed by identification by serum neutralization and immunoperoxidase technique.

3) The application of RT-PCR for identification of BEF virus to overcome problems associated with antigenic relationship of rhabdoviruses when using the conventional serological techniques for virus identification.
4) Utility of Electron microscope for confirmation of BEF virus infection in inoculated mice brain.

MATERIAL AND METHODS

1. Animals:

A Governmental farm consists of two parts one dairy and the other is fattening.
Animals under investigation during 2000 were 790 dairy and 365 beef.
Animals under investigation during 2001 were 745 dairy and 355 beef.
Animals under investigation during 2004 were 564 dairy and 136 beef.

2. Blood samples:

Heparinized blood samples were collected from animals during febrile phase. The buffy coat was separated and washed three times with phosphate buffered saline (Nagano et al., 1990). Buffy coat was used for virus isolation in suckling mice and detection of virus nucleic acid by reverse transcriptase-polymerase chain reaction (RT-PCR).

3. Virus isolation :

Virus isolation was carried according to Nandi and Negi, (1999) as the buffy coat sample from individual animal (100 ul) were inoculated intracerebrally into three suckling mice (2-day-old). Following virus propagation, brain impression smears from experimentally inoculated mice were used for detection of BEF virus antigen by IP staining. The brain tissue was a used for detection of BEF virus nucleic acid by RT- PCR.

4. Immunoperoxidase technique:

Mice brain impression smears were fixed with 10% acetone for 10 minutes. Cell culture (VERO & BHK) were fixed by heat for 4 hours at 80 C. The endogenous peroxidase was exhausted by 0.3 % hydrogen peroxide for 10 minutes. The slides were flooded with 3% bovine serum albumin and incubated for one hour at room temperature for blocking. The films were flooded with rabbit anti-BEF virus antibodies (working dilution 1: 100) and incubated at room temperature for one hour. The slides were washed 3 times with PBS- tween. The slides were flooded with anti-rabbit peroxidase conjugated immunoglobulins (diluted 1: 1000 in PBS-tween) and incubated at room temperature for one hour. Washing was repeated as mentioned before. The substrate was prepared by solubilizing 2 mg of 2 amino 9 ethylcarbazole (AEC) in 0.3 ml dimethylformamide. The substrate was diluted in 5 ml of 0.05M sodium acetate buffer (pH, 5.5) and 25 ul of 3 % hydrogen peroxide were added. After 15 minutes incubation at room temperature, slides were washed 3 times with PBS and finally by distilled water. The slides were examined by light microscopy (Zaghawa, 1989).

5. Detection of BEF virus nucleic acid by reverse transcriptase-polymerase chain reaction (RT-PCR):

5.1. Extraction of RNA:

The buffy coat was extracted with an equal volume of chloroform and the aquous phase was collected by centrifugation at 14,000 rpm at 4 C for 15 minutes. Sodium dodecyl sulphate (Sigma chemical Co.) and proteinase K (Sigma chemical Co.) were added to give a final concentration of 0.05% and 1.0 mg/ml, respectively. Following incubation at 37 C for 1 hour, the samples were extracted with an equal volume of acid phenol with pH of 4.3 (Amersham Co.) and then with chloroform: isoamyl alcohol (24:1). The RNA was precipitated with 2.5 volumes cold ethanol. The RNA was collected by centrifugation at 14,000 rpm for 20 minutes. The RNA was suspended in 100 ul of 90% dimethyl sulfoxide solution at 95 C for 5 minutes (Rolfs et al., 1992).

5.2. Reverse transcription (RT):

Ready to go RT-PCR beads (Pharmacia) were dissolved in 18 ul bidistilled water. The RNA was denaturated at 95 C for 5 minutes and 2 ul were used in reverse transcription (RT) reaction. Random oligonucleotide primer was used for synthesis of a complementary DNA (cDNA). The reaction mixture was incubated at 42 C for 15 - 30 minutes and then at 95 C for 5 minutes for inactivating reverse transcriptase (Sambrook et al., 1989).

5.3. Polymerase chain reaction (PCR):

PCR was conducted using 2.5 units Taq DNA polymerase in a reaction buffer containing 50mM Kcl, 10 mM tris-HCL (pH, 9.0) and 200 uM each dNTPs. The sequences of the primers were as follows: primer 1 (P1) 5' TACAACAGCAGA TAAAC'3 and primer2 (P2) 5'AAGATTCATTTGGAGAAA 3'. Thesequences of the primers was derived from the sequence at the 3' end and 5' ends of the glycoprotein coding gene (G), respectively. The amplification was done for 30 cycles in a thermal cycler (Biometra). The PCR cycle consists of denaturation at 94 C for 1 minute, annealing at 55 C for 1 minute, extension at 72 C for 1.5 minutes and the final extension of 10 minutes at 72 C (Walker et al., 1992).

5.4. Gel electrophoresis:

Following PCR amplification, the PCR products were run on 1% agarose gel in tris acetate EDTA (TAE) buffer (PH, 8.0). The gel was stained with ethidium bromide and the DNA fragment was visualized by U.V. illumination and then photographed.

5.5. Electron Microscopy of mice brain:

Electron microscopy was carried out for brain according to Kay (1965). The technique was carried out at the Unit of the Electron Microscope, Assuit University, Egypt.

Statistical analysis:

Statistical analysis was carried out according to Epi-Info computer program (1994)

RESULTS AND DISCUSSIONS

Table (1&2) describes the percentage of clinical disease during summer 2000 were 49.87 % and 31.87 % in dairy and beef cattle respectively. The mortality rates were 2.66 % and 2.19 % in dairy and beef cattle respectively. During summer 2001 the percentage of clinical disease

were 18.8 % and 12.67 % in dairy and beef cattle respectively. The mortality rates were 0.7 % and 0.8 % in dairy and beef cattle respectively. During summer 2004 the percentage of clinical disease were 35.6 % and 36 % in dairy and beef cattle respectively. The mortality rates were 1.5 % and 0.9 % in dairy and beef cattle respectively. Table (3) illustrated the statistical analysis of data concerning dairy cattle showed significant effect of year on both clinical disease and mortality rates. Table (4) illustrated the statistical analysis of data concerning beef cattle showed significant effect of year on clinical disease and non significant effect on the mortality rates.

It is clear that the severity of the outbreak during summer 2000 was probably due the absence of herd immunity for about 10 years as the last outbreak was in 1991 (Hassan et al.1991). The very mild outbreak during summer 2001 is actually due to the high level of herd immunity lasts from the previous outbreak. It is not closed that the virulence of the BEF virus plays a role in the severity of the disease as documented by Yeruham and Braverman (2005).

Again it is now to confirm that the level of immunity resulted from natural infection is the key for BEF morbidity and mortality percentages as in summer 2004. This is clear after 4 years absence of the infection. Retrospective analysis indicates that vectors - apparently mosquitoes - infected with BEF virus could have been over-wintering. Similar fluctuation of BEF outbreaks were described by Yeruham et al. (2002) in the Jordan Valley and by Abu Elzein et al. (1997) in Saudia Arabia. They concluded that the vector(s) is not known for certain but the available evidence indicates that mosquitoes, and not Culicoides species, are the natural vectors of BEF virus. In our situation further studies are needed to illustrate the role of insects in transmission and maintenance of the virus during the inter-epizootic period.

In these study we isolated the BEF virus from leukocytes of naturally infected cattle by intra-cerebral inoculation of baby mice (fig.1) These results are in accordance with Van der westhuizen (1967) in baby mice and Inaba et al. (1968) in suckling hamster and rat. The isolation was confirmed by IP staining in both impression smear of mice brain and inoculated cell cultures after the appearance of cytopathic effect (fig.4) which was inhibited by the addition of the BEF hyperimmune serum. The positive results of the immuneperoxidase test in brain impression smears (fig.2) and in inoculated cell culture (fig.3) are quite clear for confirmation of the BEF diagnosis. Immunohistochemical staining of impression smears from the infected mice brain proved to be a simple, rapid and sensitive method for identification of BEF virus (Khalil et al. 2001).

The serological identification of rhabdoviruses is considered a field diagnostic problem due to the antigenic relationship between the members of this group. The BEF virus shares with rabies virus in many epitopes especially the neutralizing one (Calisher et al., 1989). Subclinical infection of

animals with rabies-like viruses may interfere with the conventional diagnosis of BEF virus. For these reasons, the development ofa recent, sensitive and specific methods to identify a specific nucleic acid fragment of BEF virus is essential.

We have studied the sequence analysis of BEF virus from the available literatures (Kongsuwan et al., 1998). Thise nable us to choose two suitable oligonucleotide primers for amplification of BEF virus genome by using RT-PCR. The RT-PCR products yielded a clear single band on agarose gel stained with ethidium bromide. The amplified DNA fragment corresponded to 500 base pairs (bp) in length which was expected from the known sequence of BEF virus. This indicated a positive and specific results. Moreover, no differences in the size of bands of RT-PCR product were detected on agarose gel. These results indicated that there was no deletion or insertion mutation within the region flanked by the two primers (Glycoprotein coding gene). The application of RT-PCR to confirm the diagnosis of BEF virus outbreak was found to be very sensitive, specific and of value for rapid diagnosis of the disease. This is essential to carry out the suitable control program in order to minimize the economic losses.

RT-PCR has many advantages as it was possible to detect as little as two fragments of viral RNA from infected tissue by ethidium bromide staining after 30 cycles of PCR (Wu et al. 1992). There is no need for virus propagation or purification, moreover, RT-PCR is not time consuming since all procedures involved take about 6 hours to be completed (Davis and Boyle, 1990).

Fig. (5) showed the detection of bovine ephemeral fever virus particles in ultra thin section of mice brain by electron microscope as cone shape or sometimes circular when cut out. The electron microscope was used for BEF virus diagnosis by Chiu and Liu (1984) in Taiwan describing the general morphology of rhabdoviruses. At the local regional situation it is a good diagnostic tool for confirmation of the diagnosis of BEF virus infection

It can be concluded that:

1. *The retrospective analysis of the data concluded that the level of herd immunity resulted from natural infection and the virulence of the BEF virus affect the severity of the outbreak.*
2. *Immunoperoxidase is a sensitive and specific method for detection of BEFV antigen in brain of inoculated mice and infected cell cultures.*
3. *The application of RT-PCR to confirm the diagnosis of BEF virus outbreak was found to be very sensitive, specific and of value for rapid diagnosis of the disease*
4. *The application of Electron microscope for BEF diagnosis is another method to confirm the diagnosis of the disease.*
5. *Further studies are needed to produce commercially a genetically engineered vaccine for control of the disease.*

Acknowledgement

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Table (1) : Retrospective clinical investigation of BEF in Governmental Egyptian farm during 2000, 2001 & 2004

Year	Dairy			Beef		
	No. of Examined Animals	No. of Diseased Animals	Percentage	No. of Examined Animals	No. of Diseased Animals	Percentage
2000	790	394	49.87 %	365	116	31.78 %
2001	745	140	18.8 %	355	45	12.67 %
2004	564	209	37.05 %	136	49	36.02 %

Table (2): Mortality rate in dairy and beef cattle in Egyptian Governmental farm during the outbreaks of 2000, 2001 & 2004

Year	Dairy			Beef		
	No. of Examined Animals	No. of Dead Animals	Percentage	No. of Examined Animals	No. of Dead Animals	Percentage
2000	790	21	2.66 %	365	8	2.19 %
2001	745	5	0.67 %	355	3	0.8 4%
2004	564	5	0.88 %	136	2	1.47 %

Table (3). Statistical analysis of data concerning the effect of year on the clinical disease and mortality rate in dairy cattle

EFFECT OF YEAR ON CLINICAL DISEASE	EFFECT OF YEAR ON MORTALITY RATE
YEAR D+VE D-VE TOTAL 2000 394 396 790 2001 140 605 745 2004 209 355 564 TOTAL 743 1356 2099 Chi-Square 162.9056 P <.0001	YEAR DEAD ALIVE Total 2000 21 769 790 2001 5 740 745 2004 5 564 569 Total 31 2073 2104 Chi-Square 12.3287 P< 0.0021
Number of diseased dairy animals differed significantly among years.	Number of mortal dairy animals differed significantly among Years.

Table (4). Statistical analysis of data concerning the effect of year on the clinical disease and mortality rate in beef cattle

EFFECT OF YEAR ON CLINICAL DISEASE				EFFECT OF YEAR ON MORTALTY RATE			
YEAR	D+VE	D-VE	Total	YEAR	DEAD	ALIVE	Total
2000	116	249	365	2000	8	357	365
2001	45	310	355	2001	3	352	355
2004	49	87	136	2004	2	134	136
Total	210	646	856	Total	31	2073	2104
Chi-Square 47.0218 ' p <.0001				Chi-Square 12.3287 P < 6.21			
Number of diseased beef animals differed significantly among years				Number of mortal beef animals did not differ significantly among years			

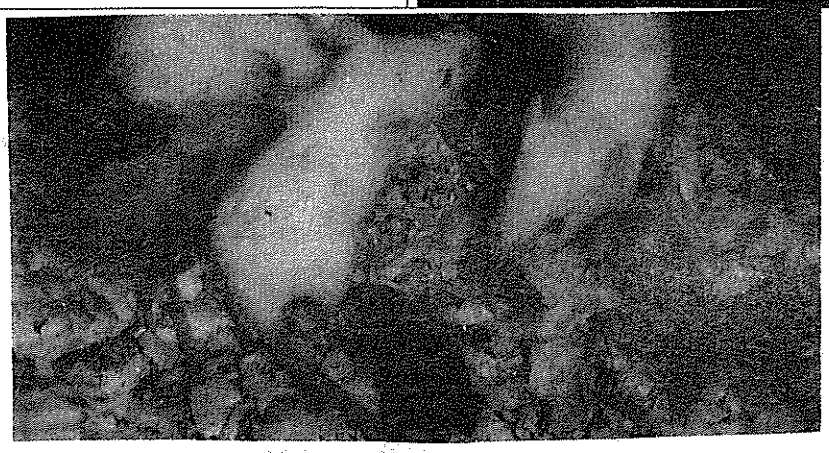

Fig. (1): Death of baby mice after inoculation with BEF virus isolate

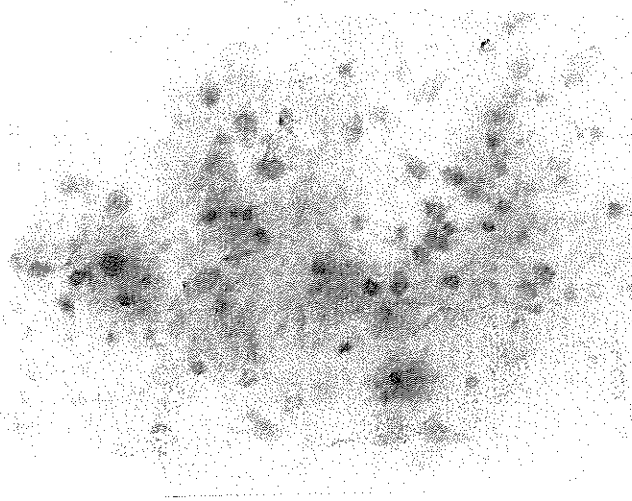

Fig. (2): Posivive immunoperoxidase showing reddish staining intracytoplasmic granules in baby mice

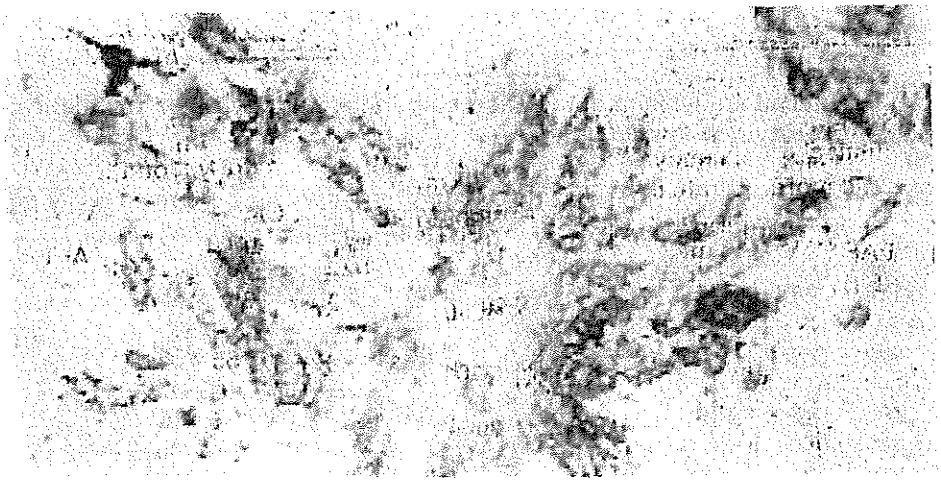

Fig. (3):Intense reddish brown staining intracytoplasmic granules as a positive immunoperoxidase test of VERO cells infected with BEF virus

Fig.(4): Cytoplasmic granulation and vasculization with synthetial cell formation as cytopathic effect of BEF virus in VERO cells

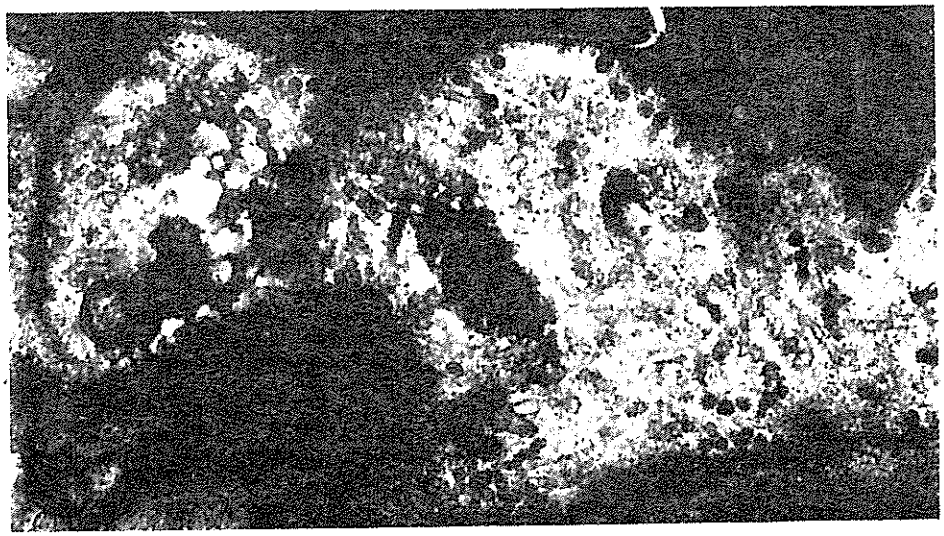

Fig. (5): Electron Microscopy of mice brain infected with BEF virus showing typical virus particles Bullet-shape in the endothelial cells of blood vessels