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DETECTION AND ISOLATION OF BOVINE RESPIRATORY SYNCYTIAL VIRUS IN BUFFALOES

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SUMMARY

The present study was carried out for the detection and isolation of bovine respiratory syncytial virus (BRSV) in 480 buffaloes (calves, feedlot and dairy buffaloes) that showing respiratory manifestations (n=205) or apparently normal (n=275). The samples were collected between August 1997 to May 1999 from ten dairy farms distributed in nine Egyptian governorates. 480 sera samples were collected and examined for detection of specific BRSV antibodies using ELISA. 205 nasal swabs were collected from diseased animals and used for virus detection (by dot ELISA) and isolation. Viruses were isolated on vero cell line and identified by monoclonal enzyme-immunoassay, Dot ELISA, immune fluorescent assay (IFA) and fluorescent focus neutralizing assay. The results indicated that out of 205 nasal swabs tested, BRSV was detected in 20.5% (42/205) of the samples. Four viruses were isolated on vero cell line (two from EL-Behaira and one from each of EL-Sharkia and Ismailia Governorates). Of the 4 isolated viruses, 3 were isolated from buffalo calves less than six months old and one isolate from dairy buffaloes of 1.5 years old. The viruses were isolated from samples collected either in autumn (2 isolates) or in winter (2 isolates) seasons. The serology results using ELISA revealed that 37.5 % of the collected serum samples were positive for BRSV antibodies. ELISA proved to be the most sensitive, rapid and efficient test for identification of the virus, as well as for serodiagnosis of BRSV. This study describes for the first time the isolation of BRSV from buffaloes in Egypt.

INTRODUCTION

Bovine respiratory syncytial virus (BRSV) is one of the most important causes of respiratory diseases in calves, feedlot and dairy cattle. BRSV was identified to be economically important dis-

case of cattle associated with epidemics of acute respiratory infections allover the world (Collins et al., 1996). Respiratory syncytial virus (RSV) is a member of the genus Pneumovirus, subfamily Pneumovirinae of the family Paramyxoviridae. Isolation of RSV from human beings was first reported by Chanock and Finberg in (1957), whereas BRSV was first isolated from cattle by Paccaud and Jacquier (1970) then reported all over the world. BRSV infections and reinfections commonly occur world wide, and can cause severe respiratory symptoms in calves almost during autumn and winter (Baker, et al., 1986b). Although BRSV infections seem to be absent during summer, infection and disease annually recur in a herd, even when the herd is closed and reintroduction of the virus is unlikely (Westenbrink et al., 1985). The disease is lasting from 1 to 2 weeks (Baker et al., 1997). The morbidity rate in outbreaks of BRSV infection is high whereas, the mortality rate is variable range up to 20 % which is usually due to secondary bacterial pneumonia (Fenner et al., 1993). Many predisposing factors have been speculated to be important in occurrence of BRSV outbreaks such as weather, transportation, nutritional and breed predisposition (Baker, 1987).

Although many infectious agents (parainfluenza-3 (PI-3) virus, bovine adenovirus, bovine coronavirus, infectious bovine rhinotrachietis (IBR) virus and bovine viral diarrhea (BVD) virus) have been associated with the respiratory disease complex, BRSV may be very important as a predisposing factor, especially in older calves (Uttenthal et al., 1996). Cattle are the principle reservoir of infection, however chronic carrier state has never been demonstrated or observed in experimental infection and natural outbreaks indicated that passively derived antibody does not protect calves against BRSV infection (Fenner et al., 1993).

Detection of BRSV from cattle in Egyptian governorates was reported (Abo El-lail ,1992 and Sahar,1998). The virus was isolated from cattle for the first time by Saber et al. , 1996).

Buffaloes also suffer from BRSV infection. Only few reports have been published on the detection of specific antibodies to BRSV in serum samples in India (Bansal and Singh, 1980) and Italy (Cavirani et al.,1995 and Palomba et al. 1995). However, the virus was not isolated. In the present study, trials were conducted for detection and isolation of BRSV from buffaloes in ten dairy farms distributed in nine Egyptian governorates.

MATERIAL AND METHODS

Samples: 480 non-vaccinated male and female buffaloes aged between one month to four years were included in the study. 205 nasal swabs were collected with sterile sponge and used for detection and isolation of bovine respiratory syncytial virus (BRSV). 480 serum samples were collected

and tested for antibody levels to BRSV. Preparation of nasal secretions was carried out according to Elvander, (1996) then kept at -70°C for virus isolation. Serum samples were stored in sterile screw capped tubes and kept at - 20°C until used.

Animals: Rabbits were used for preparation of hyper immune serum against the bovine respiratory syncytial virus antigen.

Cell Line: The monkey kidney cells (VERO cells) were used for virus isolation. The cells were maintained using Eagle's minimum essential media (E-MEM) with Earlís salts, L-glutamine, and 25 ml HEPES. 10% fetal calf serum (FCS) was used in growth medium for cell propagation whereas 1-2% of FCS was used in maintenance medium for virus isolation.

Dot ELISA: The assay was carried out according to Zeng and Gan (1992) for detection of bovine respiratory syncytial virus in nasal discharges. Buffers and solutions used in Dot ELISA include: Tris buffer (pH 7.8); blocking buffer (5% NFDM in Tris buffer); diluting buffer (0.5 % NFDM in Tris containing 0.05 % Tween 20); washing buffer (Tris buffer containing 0.05 % Tween 20); Substrate solution (30 mg 4-Chloro-1-naphthol in 10 ml Pure methanol); Substrate working solution (1.7 ml Substrate solution was added to 8.3 ml of Tris buffer and 30 µl H2O2 was added before use).

Isolation of BRSV: The isolation procedure was carried as described by Elvander (1996). Vero cells (80 % confluence) in 25 ml tissue culture bottle was inoculated with 0.2 ml of the prepared nasal secretions and incubated at 37°C / 5% CO2 for 7 days with daily examination under inverted microscope for detection of the characteristic cytopathic effect (CPE) specifically the syncytia formation. Suspected samples were kept frozen at - 70°C in MEM free FCS for propagation on tissue cultures. Control non-inoculated cells were included in each step of virus isolation.

Virus titration: Virus titration was performed according to Frey and Liess, (1971).

Identification of BRSV:

Monoclonal antibodies based-ELISA: Commercial monoclonal enzyme-immunoassay was used for the detection of respiratory syncytial virus antigen in either nasal secretions or in characterization of the isolated viruses. Buffers and solutions used in this ELISA include: Washing and diluting buffer (Reagent diluting 1:9 in fresh and germ free redistill water.); Substrate solution (TMB solution [3,3,5,5-tetra-methyl-benzidine]); Stopping solution (2.5 mol/ lH2SO4). Microtiter strip wells as a solid phase were coated with specific monoclonal anti-RSV antibodies. 50 µl of each of isolates and other random smears of nasal secretions were pipetted into 28 wells of the plate, 50 µl of positive control working solution pipetted into 1 well and 50 µl of negative con-

trol working solution were pipetted into each of two wells . One well was left for substrate blank (A1). The plate was incubated for1 hour at 37°C then washing was applied 5 times with 250 µl of working washing solution . 50 µl of anti-RSV antibody - biotin conjugate working solution were dispensed into all wells except A1 and incubated for 1 hour at 37°C then washed again as before. 50 µl of streptavidin - peroxidase conjugate working solution were dispensed into all wells except A1 and incubated for 30 minutes at 37°C and repeated washing procedure was performed. 50 µl of ready- for- use TMB solution was dispensed into all wells and the plate kept in dark area for15 minutes at room temperature (20 to 25°C). 50 µl of stopping solution was dispensed into all wells and optical density was measured by ELISA microplate reader at 450 nm . Dual wavelength reading using 620 nm as reference wavelength was recommended . Absorbance value of positives were more than 20 % above the mean absorbance value of the 2 negative control were calculated.

b) **Dot ELISA:** This assay was performed as described above on the 28th nasal smears including the four isolates.

c) **Immunofluorscent assay (Direct fluorescent antibody technique)**: The technique was performed according to Nettleton and Herring, (1983). The same 28th nasal samples including the isolates were assayed using cell culture staining chamber (CCSC). Buffers used in immunofluorescence (FAT) were as follows: Washing and diluting buffers (PBS pH 7.4); Mounting buffer (90 % glycerin in PBS); Fixation mixture (60.0 ml Acetone, 25.0 ml Ethanol, 15.0 ml Methanol).

D) **Fluorescent focus neutralisation assay:** The assay was carried out according to (Huang et al., 1992). This assay was used for identification of the local isolates. After mixing the isolated viruses with reference anti-BRSV antibodies, the mixture was inoculated in cell culture and incubated for 48 hr and assayed using the IFA. Reduction in the flourescine signals from the inoculated cells by more than 70% compared with the control inoculated cells by the same isolates and the BRSV reference strain was considered positive for neutralization.

Serology:

Indirect ELISA: This assay was carried out according to Rhodes et al.,(1989) for detection of bovine respiratory syncytial virus antibodies in serum samples of buffaloes. Buffers and solutions used in solid phase ELISA were as follows: Coating buffer (carbonate bicarbonate buffer, pH 9.6); blocking buffer (5 % Non fat Dry milk (NFDM) in PBS containing 0.05 % tween 20); washing Buffer (0.05 % tween 20 in PBS); Diluting Buffer (0.5 % NFDM in PBS containing 0.05 % tween 20); Stopping solution (5 % SDS in distilled water).

RESULTS

Detection of BRSV in nasal discharges of buffaloes in relation to locality, age and season using Dot ELISA:

Table (1) Shows the data obtained from nine governorates in different seasons at the time from August,1997 to May,1999. The samples of nasal secretions were collected from buffaloes at different ages started from one month up to 4 years and examined for detection of BRSV using Dot ELISA. Forty two buffaloes suffered from respiratory infection and the percentage of BRSV was 20.5 % for all nasal samples (Figure 2). It was observed that most of positive samples were collected from buffalo calves 1-6 months in age and in winter and autumn seasons.

Virus Isolation:

Isolation of the virus on vero cells was carried out from positive nasal swabs based on the results obtaind using Dot ELISA. The inoculated samples were examined daily for the presence of cytopathic effects (CPE). The positive samples showed the characteristic syncytial cells. CPE was obvious 5-7 days post-inoculation and gradually increased till 80 % of the cell sheet was completely detached. Table (2) shows that only four viruses out of 42 nasal swab samples could be isolated from samples collected from Ismailia, El-Sharkia and El-Behaira governorates, while nasal swabs collected from El-Gharbia; El-Giza ; El-Fayowm ; Beniswaf ; El-Seuz and El-Dakahlia were negative for virus isolation. Three viruses were isolated from nasal discharges of buffalo calves aged from 1 up to 6 months while the fourth isolate was isolated from 1.5 year old buffalo.Two viruses were isolated from El-Behaira governorate in winter while one virus was isolated from each of El-Sharkia and Ismailia governorates in autumn..

Titration of the isolated viruses:

The four isolates which showed clear CPE were titrated after eight passages on the Vero cell line and the infectivity titer was calculated and expressed in log10 TCID50/ml according to the method of Reed and Muench, (1938).The obtained titers were shown in Table (3). It was clear that the maximum titer measuring $10^{5..3}$ TCID50 / ml was obtained from an isolate collected from Ismailia governorate while the minimum titer was $10^{2..5}$ TCID50 / ml which was obtained from El-Sharkia governorate.

Identification of the isolated viruses:

a) **Monoclonal antibodies based ELISA:**

Microtiter strip wells (solid phase) were coated with specific monoclonal anti-RSV antibodies and used for identification of the isolated viruses. The deep yellow represent the positivity of the utilized ELISA were observed in the wells contained the four isolated viruses (Figure 2).

Table (1): Detection of BRSV in nasal discharges of buffaloes in relation to locality, age and season using Dot ELISA.

Governorate	Date of sample collection	No. of Nasal swabs	No. of positives	Age		Seasons
				Month	Year	
El-Gharbia	1997	10	2	9		Summer
El-Giza	1997	36	6	1-6		Autumn
		7	0		2-3	
El-Fayowm	1998	13	5	3-6		Spring
		7	1		2	
Beni Suaif	1998	8	1		2	Summer
El-Ismailia	1998	20	5	3-6		Autumn
		11	3	6-12		
El-Sharkia	1998	25	6	2-6		Autumn
		13	3		1.5-2	
El-Behaira	1998	16	5	1-6		Winter
		14	3		1-2	
El-Suez	1998	6	2	6-12		Winter
		1	-		3-4	
El-Dakahlia	1999	8	-	6-12		Summer
		10	-		1.5-2	
Total		205	42			

b) **Dot ELISA:** The four tissue culture adapted isolates were positive when tested by Dot ELISA.

c) **Immunofluorescent assay (IFA):** The direct FAT was performed for identification of the isolated viruses using fluorescein isothiocyanate conjugated BRSV antisera. The inoculated vero cells showed faint green yellowish fluorescence (Figure 1) compared with the non-inoculated cells which were free from any fluorescince (Figure 1). BRSV was detected in the four isolates. Control positive virus and standard antisera were also inclued in the test.

d) **Fluorescent focus neutralization assay**: This

Table (2): BRSV Virus isolation from nasal swabs on Vero cells.

Year of samples collection	Governorate	No. of Positive wabs by dot EISA	No. of Isolate	Age	
				Month	Year
1997	El-Gharbia	2	-	6-12	-
1997	El-Giza	6	-	1-6	-
1998	El-Fayowm	5 1	- -	3-6	2-3
1998	Beni Suaif	1	-	-	2-3
1998	El-Ismailia	5 3	1 -	2-6 7-12	-
1998	El-Sharkia	6 3	1 -	1-6	1-2
1998	El-Behaira	5 3	1 1	1-6	1-2
1998	El-Suez	2	- -	6-12	-
1999	El-Dakahlia	-	-	-	-
Total		42	4	-	-

Table (3): Titration of BRSV isolated from nasal discharge and adapted on Vero cells.

Governorates	El-Behaira		El-Sharkia	El-Ismailia
Code no. of isolates	Behaira 9/98	Behaira 13/98	Sharkia 46/98	Ismailia 103/98
Titers of isolates expressed in LOG_{10} $TCID_{50}$ ml.	$10^{3.3}$	10^{5}	$10^{2.5}$	$10^{5.3}$

Table (4): Comparison between ELISA; Dot ELISA and IFA in detection and identification of BRSV antigen in nasal discharges including isolates

Governorate	Cod No. of Nasal Swabs	MAB-ELISA		DOT- ELISA		IFA	
		+ve	%	+ve	%	+ve	%
El-Gharbia	1 14 20 28	- - - -	0/4 (0)	- - - -	0/4 (0)	- - - -	0/4 (0)
El-Giza	5 14 26 41	5	1/4 (25)	5	1/4 (25)	5	1/4 (25)
El-Fayowm	9 12 18 23	- - - -	0/4 (0)	- - - -	0/4 (0)	- - - -	0/4 (0)
El-Ismailia	103 113 126 128	103	1/4 (25)	103	1/4 (25)	103	1/4 (25)
El-Sharkia	45 46 50 57	46	1/4 (25)	46 57	2/4 (50)	46	1/4 (25)
El-Behaira	4 7 9 13	4 9 13	3/4 (75)	4 9 13	3/4 (75)	9 13	2/4 (50)
El-Suez	1 2 8 16	2	1/4 (25)	16	1/4 (25)	- - -	0/4 (0)
Total	28	7	25	8	28.5%	5	17.8%

Table (5): Detection of BRSV antibodies in serum samples in relation to animal age using indirect ELISA

Age of animal	No. of serum samples	ELISA			
		+ve	%	+ve	%
Calves (1-3 months)	120	24	20	96	80
Calves (3-6 months)	118	78	66.1	40	33.9
Feedlot (6-9 months)	55	28	50.9	27	49.1
Feedlot (9-12 months)	63	18	28.6	45	71.4
Dairy buffaloes (4-12 months)	124	32	25.8	92	74.2
Total	480	180	37.5	300	62.5

assay was carried out to identify the viral isolates as specific anti-BRSV antibodies were used to neutralize the viral antigens of the isolated viruses resulting in reduction of the fluorescent foci (the stained cells inoculated by these viruses). It was shown that over 80% reduction in the fluorescenct foci of the vero cells inoculated by the viral isolates antisera mixture after incubation compared to the cells inoculated with the same isolate alone.

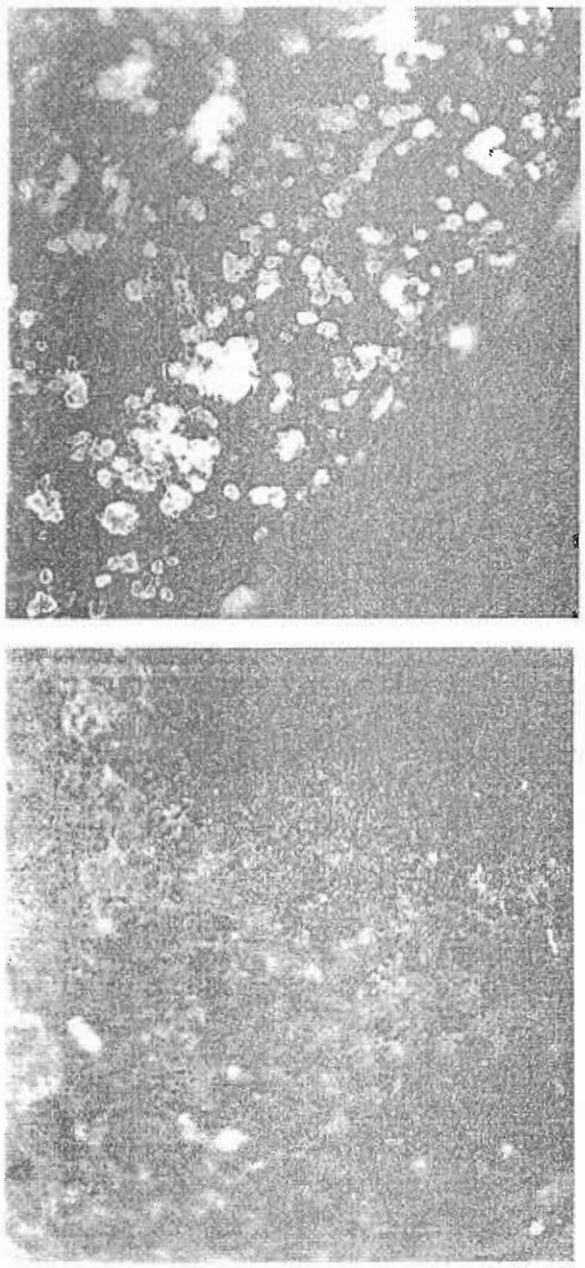
Comparison between monoclonal antibodies based ELISA); Dot ELISA and IFA in detection and identification of BRSV antigens in nasal secretions :

Twenty eight random nasal swabs were collected from buffaloes in seven governorates (four from each governorate) for detection of BRSVantigens by using the three tests. Regarding the both monoclonal enzyme-immunoassay (Kit ELISA) and Dot ELISA, the highest positivity for BRSV antigens were detected in samples collected from El-Behaira governorate (75%) while the lowest positivity were detected in samples collected from El-Giza, El-Seuz and El-Ismailia (25%). The four isolates were positive for identification by the three tests. Immunofluorescent assay was the lowest in detecting the positive samples (5 positives) than Kit ELISA (7 positives) and Dot ELISA (8 positives). Samples collected from El-Gharbia and El-Fayowm were negative for BRSV

antigens by the three tests (Table 4).

Detection of BRSV antibodies in serum samples in relation to animal age using indirect ELISA:

ELISA was used for demonstration of specific antibodies against BRSV in serum samples. It is evident from the results shown in table (5) that the highest percentage of ELISA positive (66.1%) identified within the age group from 3 to 6 months while the lowest percentage of ELISA positive (20% and 25.8%) was found within the age groups less than 3 months and more than 12 months, respectively.



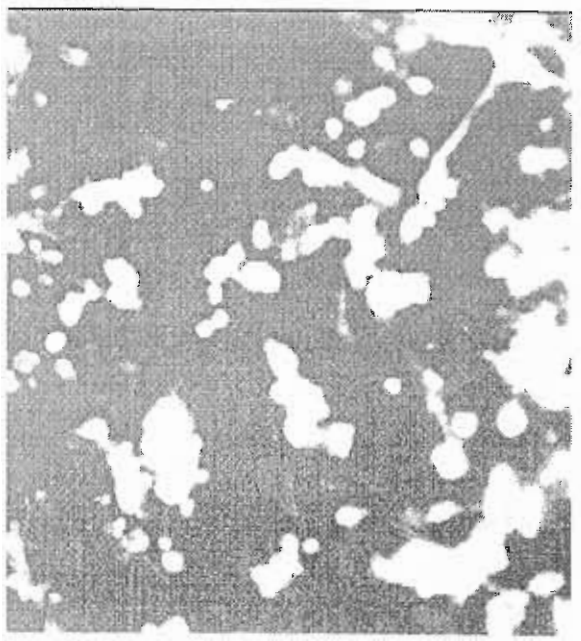


Figure 1: Flourescine - positive vero cells inoculated by 2 of the isolated viruses on the top photos and the control non inoculated cells on the bottom photo.

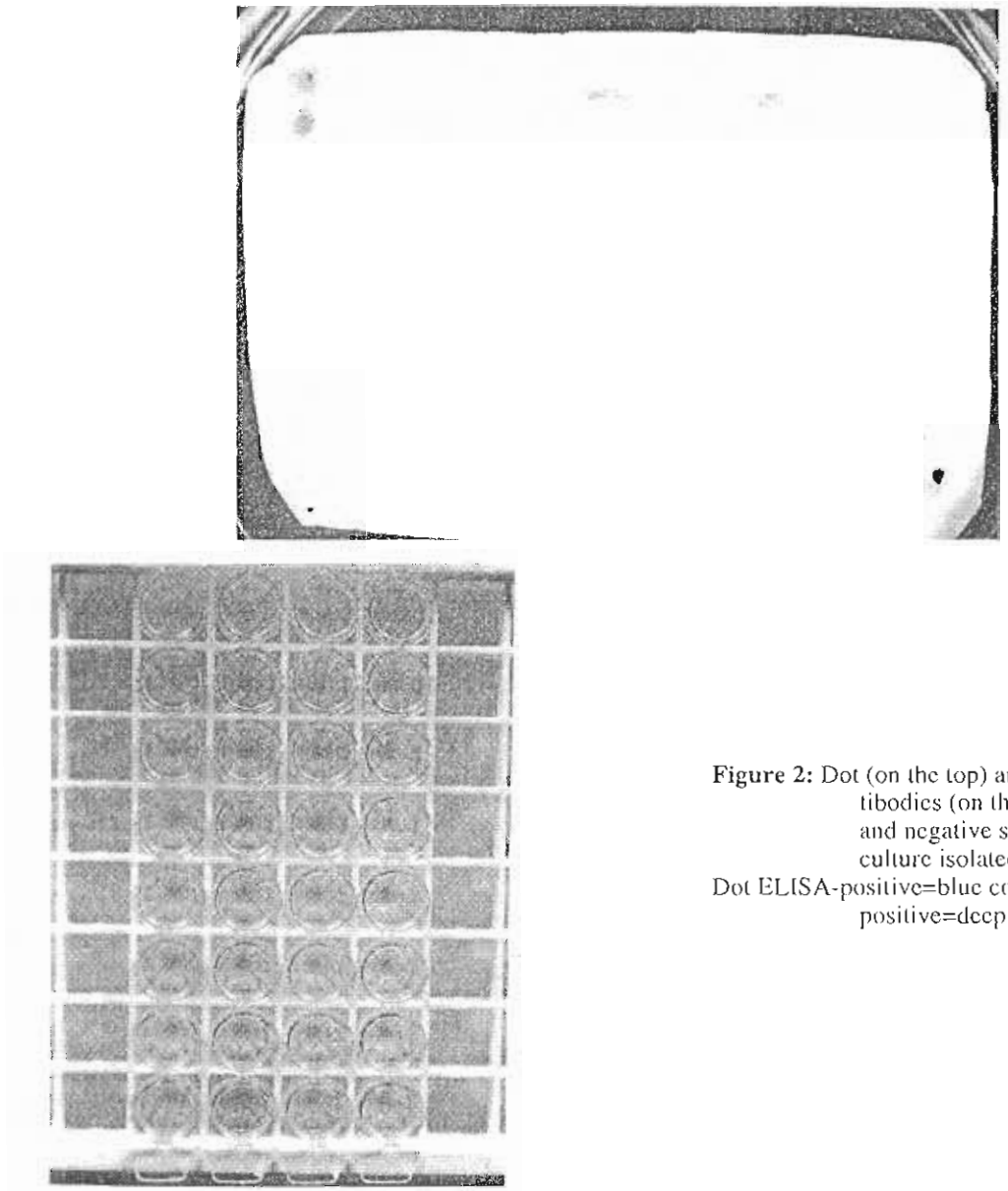


Figure 2: Dot (on the top) and monoclonal based antibodies (on the Left) showing positive and negative samples (nasal and cell culture isolated viruses).
Dot ELISA-positive=blue color Mab-ELISA-positive=deep yellow color

DISCUSSION

Bovine respiratory syncytial (BRSV) is one of the most important causes of the respiratory tract disease in cattle especially, in young calves, in many countries. The disease is responsible for economic losses that may be directly related to death of the animal due to severe interstitial pneumonia or indirectly related to the unthriftness, delayed marketing, and treatment costs (Evermann et al.,1985).

The present investigation includes trials for detection, isolation and identification of BRSV from 205 buffaloes in different ages suffering from respiratory manifestations, and serological screening for the presence of BRSV antibodies in the serum collected from 480 diseased and appa-

rentlly healthy buffaloes in different localities in Egypt.

The crowdness and bad hygienic measures play a role and predispose in wide spread infection of this infectious disease in animal population (Healy et. al., 1993). The finding in this investigation that 205 buffaloes from 480 (42 %) were suffering from respiratory manifestation may be due to many factors. Such situation of clinical manifestations were previously reported by Pirie et al., (1981) and Baker et al., (1997).

Although it is still difficult to demonstrate the virus through isolation procedures, the ability to diagnose BRSV infections has improved through the development of tests such as immunofluorescent antibody staining and antigen detection enzyme-immunoassay (Collins et al., 1996 and Baker et al., 1997). Therefore ,application of the Dot ELISA in this study for the detection of BRSV antigens in nasal discharges was successful in detecting 42 of the 205 collected samples (20.5%) and most of the positive samples (number=32) were collected from calves below 6 months of age (Table 1). The obtained results are similar to those obtained by Pirie et al., (1981) and Kimman et al., (1988). The epizootiological data obtained from nine governorates are shown in table (1).

The positive forty two nasal swabs by Dot ELISA from diseased animals of different ages from nine governorates were processed for an attempt of virus isolation (Table 2). Only four viruses (showed typical CPE in the form of syncytial appearance at 5-7 day post-inoculation) were isolated from nasal swabs. The isolation was achieved only in three governorates i.e. El-Behaira, El-Sharkia and Ismailia. The low rate for isolation of BRSV was common and reported by others (Yamashita et al., 1985). This lowness may be due to the fact that the virus is labile outside the host and is thought to lose viability during transport to the laboratory and is thermolabile hence sensitive to freezing-thawing (Baker et al., 1986a ; Anderson et al., 1986 ; Baker et al., 1997). In relation to the age of the animal, the highest number of virus isolation (3 isolates) was found in calves 1-6 months of age which is considered the common age of animal susceptibility (Gershwin et al., 1989).

The majority of outbreaks due to BRSV occur in early winter (Pirie et al., 1981 and Ploeger et al., 1986) but severe epidemics may also occur during the summer months (Bohlender et al., 1982). In this study, most of the positive samples were collected in winter and autumn. The reson of that may be attributed to weather changes, increased population density and poor ventilation, and other contributed factors that affect the respiratory tract in buffaloes, however, BRSV is believed to be spread by aerosol transmission (Baker and Frey, 1985).

The four BRSV local isolates were further propagated in vero cells for measuring the infectivity titers and a maximum titer yield $10^{5.3}$ TCID50 / ml was obtained from the isolate of Ismailia governorate while the minimum titer yield $10^{2.5}$ was obtained from the isolate from El-Sharkia governorate (Table 3).

Antigenic characterization was applied to characterize the BRSV local isolates. Monoclonal antibodies-enzyme immunoassay (Kit ELISA), Dot ELISA, immuno fluorescent antibody technique (IFA) and fluorescent focus neutralization assay (FFNA) were used. The four local isolates revealed positive results for the presence of BRSV by all utilized tests confirming their characterization .

To spot out the most reliable and efficient test for detection of BRSV antigen in random nasal discharges, three tests (Monoclonal enzyme-imunoassay, Dot ELISA and IFA) were compared. As shown in Table 4, it is clear that 7 and 8 out of the 28 samples were positive (25% and 28.6%) by the Mab-ELISA and Dot ELISA, respectively. Whereas only 5 out of 28 samples (17.9%) were positive by IFA. ELISA showed considerable promise as a diagnostic tool. The assay was specific and sensitive for the BRSV identification, economic and fact as it can be performed in four to six hours, allowing a rapid diagnosis to be made. In addition, it showed its cabability to detect traces of the viral protein in the later stages of the infection while IFA require intact viral particles and intact cells to identify such amount of virus. It should noted that the obtained results was similar to those obtained by Anderson et al., (1986) and Ellis et al., (1996) and differed from studies for detection and isolation of BRSV using fluorescent antibody technique (Abo El-Lail, 1992 and Avraham et al., (1994).

ELISA as a diagnostic test for detection of BRSV serum antibodies has several technical advantages including: less laborious to perform, does not depend on cell culture and is easy to standardize. For these reasons ELISA appears to be usefulextension of the available serological tests for RBSV, particularly in a routine setting where large number of samples have eto be handled (Westenbrinl et al., 1985).

The virus and hyper immune anti-BRSV serum used for examination of 480 serum samples by ELISA test were locally prepared. One hundred and eight (37.5%) out of 480 collected serum samples showed positive BRSV antibody reactors (Table 5). The highest percentage (78%) of BRSV antibodies positive sera was observed in calves between 3 to 6 months old and the lowest percentage of positivity was present among dairy buffaloes of more than one year old . Severe infections were most often observed in calves from one to three months of age. This result is supported by the results of Kimmman et al

(1987) who indicated that firstly, the young age of the calves may determine the susceptibility to disease; secondly, the moderate levels of maternal antibodies may possibly aggravate the disease by an immunopathological mechanisms and thirdly, the presence of maternal antibodies may suppress systemic and local antibody responses and interfere with clearance of the infection. Also maternal antibodies may severely suppress the antibody responses of all isotypes after infection. Indeed, the detected anti-BRSV antibodies indicated the exposure of those animals to the infection with BRSV.

In conclusion, the present study report that BRSV infection is present in Egypt among buffaloes especially in calves. Also it affects the feedlot and dairy buffaloes and this is the first record for isolation of BRSV from buffaloes in Egypt and all over the world. Although the minor percentage of identified viral isolation, outbreaks of disease associated with the virus occur annually, usually reaching their peak during the early housing season i.e. in the months of October, November and December. It is also clear that most isolates from calves less than 3 months old. Serological investigation in the obtained results revealed that the ELISA technique is the potent serological test in routine detection of BRSV and serodiagnosis of its specific antibodies being sensitive, specific rapid and efficient test.

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