Global Veterinaria 3 (6): 472-479, 2009
ISSN 1992-6197

Prevalence of Bovine Herpesvirus-1 in Sheep and Goats in Egypt

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Abstract: The present study was carried out for detecting Bovine Herpesvirus-1(BoHV-1) antibodies in serum samples of 1600 small ruminants (sheep and goats) reared in 12 governorates at Lower Egypt f by using indirect ELISA technique. The prevalence of BoHV-1 reactors were 25.1% of the total examined animals, with higher incidence in goats (27.6%), than in sheep (23.8%). Trials for virus isolation of BHV-1 were done in seven farms distributed throughout four governorates. A total of 384 samples (163 nasal swabs, 89 vaginal swabs, 80 faecal swabs and 52 lung tissues) were collected from sheep and goats with different ages, breeds, sex and different season for virus isolation. Out of 384 samples, 40 samples showed cytopathic viral effect, 20 of them from Kafr El Sheikh, 9 from Giza and 11 from Alexanderia Governorate. Comparative studies between different techniques used for virus identification (VNT and PCR), for comparing the sensitivity, rapidity and efficiency of these tests were carried out. Twelve BoHV-1 isolates were identified by PCR, while nine BoHV-1 isolates were identified by VNT, while six BoHV-1 isolates from nasal swabs, three from vaginal swabs and three from lung tissues. Five BoHV-1 isolates were obtained from local goats, 4 from foreign sheep and 3 from local goats. The isolation rates were higher in winter than in summer, female than male and animals 2 years and above than young animals under 1 year. It was concluded that the BoHV-1 is widely distributed among small ruminants in Egypt. The PCR is more sensitive, rapid and efficient for diagnosis of BoHV-1 infection, while ELISA technique is more sensitive for detection of BoHV-1 antibodies.

Key words: Bovine herpesvirus-1 · Infectious bovine rhinotracheitis · ELISA · VNT · PCR · Sheep · Goats · Prevalence · Egypt

INTRODUCTION

Bovine Herpesvirus-1 (BoHV-1) is a member of family Herpesviridae, subfamily Alphaherpesvirinae and is now commonly referred to as bovine herpesvirus type-1. The virus has been reported to exist in many countries of the world [1] is one of the most important emerging diseases of domestic and wild cattle and it causes huge economic losses [2,3].

The disease is characterized by clinical signs of the upper respiratory tract, such as a mucopurulent nasal discharge and by conjunctivitis. Signs of general illness are also, recorded including fever, depression, inappetance, abortion and reduced milk yield. The virus can also infect genital tract and cause pustular vulvovaginitis and balanoposthitis [3].

Sheep and goats breeding has became an increasingly important industry in Egypt and other countries worldwide for production of meat, milk, skin and wool. However, the respiratory tract diseases increased, unfortunately the causative agents are rarely determined and many viruses associated with respiratory system disease in cattle have been implicated in natural and experimental infection in sheep and goats [4].

BoHV-1 produces latent infection and persists in closed herds, under stress condition like infection with Bovine viral diarrhea virus (BVDV) and / or Border disease virus (BDV), large doses of corticosteroids and transportation, the latent virus can be reactivated resulting in recurrent clinical disease or asymptomatic viral shedding [5-7].

The first indication for the presence of BoHV-1 in sheep and goats in Egypt came from a serological investigation on a limited number of sheep followed by a large number of sheep and goats sera in Upper Egypt using micro-serum neutralization test (unpublished data). However, most of these data were based on relatively small number of sera and restricted to certain farms or

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governorates. So, it was important to have a more relevant and systematic investigation on the degree of prevalence of such important virus infection among sheep and goats in different Egyptian governorates.

The present work was planned to illustrate some seroepidemiological aspects associated with the respiratory and/ or genital symptoms in sheep and goats, this investigation includes serological screening of sera for the presence of antibodies in sheep and goats by using blocking ELISA technique with trials for isolation and identification of the causative viral agents by VNT, as well as recent technique like polymerase chain reaction (PCR).

MATERIALS AND METHODS

Animals: A total number of 384 sheep and goats (121 local breed sheep, 108 foreign breed sheep and 155 local breed goats) at different ages (one month up to 4 years).

Clinical Examination of Sheep and Goats: Sheep and goats under investigations in this study were suffering from respiratory and digestive disorders including nasal and ocular discharges, some animals developed severe conjunctivitis, rhinitis, cough, skin lesion, diarrhea and body fever in most animals. From these animals, some sheep and goats suffered from genital disorders including, vaginal discharge, vulvovaginitis and some goats were aborted in the late stage of pregnancy. The aborted animals were examined for brucellosis and other microorganisms causing abortion. Some apparently normal contact sheep and goats were also examined. These animals were representatives of seven farms distributed throughout five Egyptian governorates and Giza abattoir Table 1.

Serum Samples for Serological Studies: A total number of 1600 blood samples were collected in sterile vacutainer tubes. Blood was centrifuged at 2000 rpm for ten minutes for serum separation. Serum samples were stored in sterile screw capped vials and kept at -20°C for serological studies.

Swabs: A total number of 332 swabs (163 nasal swabs, 89 vaginal swabs and 80 faecal swabs) were collected from both clinically infected and apparently normal sheep and goats. In addition, a total number of 52 samples from lung tissues showing pathological changes were collected from sheep and goats. 50 samples were from Giza Abattoir and 2 samples from El Karada farm. Swabs were collected on Hank's balanced salt solution, centrifuged at 3000 rpm for 30 minutes at 4°C and then the supernatants were collected, labeled and stored at - 70°C for virological examination.

Table 1: Distribution of sheep and goats , types f samples collected for vius isolation and serodiagnosis

			Type of samples				Animal species						
											No. of serum		
Governorates	Farms	Number of samples	Nasal swabs	Vaginal swabs	Faecal swabs	Lung tissue	Local goats	Local sheep	Foreign sheep	Governorates	Sheep	Goats	Total
1-Kafr	El-Karada	12	12	-	-	-	-	-	12	Alexandria El-Sheikh	60	20	80
		13	7	-	4	2	-	-	13	Sharkia	175	65	240
		24	15	-	9	-	-	-	24	Beheira	60	20	80
		25	19	6	-	-	-	-	25	North Sinia	117	123	240
	Sakha	10	10	-	-	-	6	4	-	Qalioubia	140	20	160
		35	15	20	-	-	15	20	-	Kafr EL-Sheikh	125	35	160
		27	10	-	17	-	13	14	-	Garbia	140	20	160
		30	30	-	-	-	16	14	-	Dakahlia	85	75	160
2- Giza	Oseam	22	5	-	17	-	6	4	12	Damietta	32	48	80
		13	-	-	13	-	-	13	-	Menofia	62	18	80
	Private farm	16	16	-	-		9	-	7	Ismailia	40	40	80
		32	10	22	-		12	10	10	Giza	60	20	80
	Giza Abattoir	25	-	-	-		15	7	3	Total	1096	504	1600
		25	-	-	-		12	11	2				
3- Alexandria	Borg El- Arab	26	-	20	6		26	-	-				
		15	-	9	6		15	-	-				
4- Qalioubia	Kafr Shouker	11	11	-	-		-	11	-				
5- Beheira	Wadi El- Natron	23	3	12	8		10	13	-				
Total	-	384	163	89	80		155	121	108				

Lung Tissues: 10% suspension of lung tissues showing clear pathological changes was made and then centrifuged at 3000 rpm for 30 minutes at 4°C. The supernatant fluid was collected in sterile screw capped vials, labelled and stored at - 70°C for virological examination.

Detection of BHV-1 Antibodies in Sheep and Goats Sera: Blocking ELISA depending on BoHV-1 gB to detect antibodies in serum samples, obtained from (Synbiotics Europe, Lyon, France) was used following the instructions of the manufacturers. Serum samples were analysed in duplicate and the average was used to calculate the competition percentage. The cut off was fixed to 45% of competition.

Virus Isolation: According to Kahrs [8] all samples were submitted to virus isolation using the MDBK cells free from BVDV infection (supplied by the Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo.). Non-infected cells were used as negative control of virus isolation and positive results were determined by the cytopathic effect resulted from cell infection. Positive samples were kept at - 70°C for virus identification and characterization.

Identification of the Virus Isolates by Virus Neutralization Test (VNT): According to the method described by Brian and Hiller [9] virus isolate were identified in presence of MDBK cell line and reference BoHV-1 positive and negative serum supplied by the Central Veterinary Laboratory UK and used for ELISA and titration of prepared BoHV-1 antigen. The plates were incubated again for 2-3 days and examined daily for the presence of cytopathic effect and titres were calculated according to the method of Mylissa [10].

Detection of Viral Nucleic Acids

Extraction of Viral DNA: According to the method described by Mylissa [10], MDBK cells sheet infected with reference BHV-1 and virus isolates were freezed and thawed 3 times, centrifuged at 3000 rpm for 5 minutes at 4°C to remove cell debris. 1% of SDS and 300 ug/ml proteinase K were added and each mixture was incubated over night at 50°C. After incubation, phenol was added to remove proteins and obtaining the DNA in pure form. A mixture of phenol: chloroform : isoamyle alcohol in a ratio of (25:24:1) was added, then with chloroform: isoamyle alcohol (24 : 1). The mixture was centrifuged at 5000 rpm for 5 minutes at 4°C and carfully the aqueous phase was transferred with wide- bore pipette to a fresh tube. The DNA was precipitated using chilled absolute ethanol, equal volume of 0.3 M sodium acetate was added and the mixture was kept at -20°C over night. DNA was pelletted by centrifugation at 14.000 rpm for 30 minutes. The DNA pellet at the bottom of the tube was washed with 70% chilled ethanol, dried then resuspendeded in 25 ul of TE buffer. Finally, 2µl containing 4 units of RNase was added and the mixture incubated at 37°C for at least 1 hour.

Determination of Isolated DNA: Extracted DNA was determined by using UV spectrophotometer at 260 nm and 280 nm wave length to measure the concentration of DNA and to determine the degree of purity.

Amplification (PCR): According to Engelenburg *et al.* [12] in 50 ul reaction tube a mixture containing 2 units of Taq polymerase, 5ul PCR buffer, 0.2 mmol deoxinucleotides, 0.1 pmol from each primer and 5ul of extracted DNA was prepared. The primer sequences is based on the seqence of BoHV-1 glycoprotein 3 (gp 3) gene according to Fitzpatrick *et al*. [13]. The specific primers were synthesized using DNA synthesizer in the Institute of Molecular Biology and Genetic Engineering , Agriculture Research Center, Egypt. The sequence of oligonucleotides are (P1 5' - CTG CTG TTC GTA GCC CAC AAC G - 3' and P2 5' - TGT GAC TTG GTG CCC ATG TCG C - 3') Primer P1 is identical to the sence strand from nucleotide 763-785 and primer P2 is identical to the antisense strand from nucleotide 935-913. Amplification reactions were performed in a thermocycler under the following conditions: a denaturation step of 1 min at 94°C, followed by 38 cycles of 1 min at 95°C (denaturation); 1 min at 60°C (annealing); 1 min at 72°C (extension) and one last extension step of 10 min at 72°C. The products were analyzed by 1% agarose gel electrophoresis stained with ethidium bromide (0.5 µg/mL) in TBE buffer pH 8.4 (89mM Tris; 89mM boric acid; 2mM EDTA) and visualized under UV light. The size of the amplified products (amplicon is 173 bp) was determined by comparison with a 100 b DNA ladder (Invitrogen). negative and positive controls were included.

RESULTS

Serological Studies on the Prevalence of BoHV-1 Antibodies in Sera of Sheep and Goats: Results of examination of 1600 serum samples collected from sheep and goats located at 12 governorates in Lower Egypt were tested serologically by blocking ELISA technique Table 2.

Table 2: prevalence of BoHV-1 specific antibodies among sheep and goats in 12 governorates in Lower Egypt

	Sheep			Goats			
Governorates	Total No.	No. of+ Ve	% of+ Ve	Total No.	No. of+ Ve	% of + Ve	Overall percentage¤
Alexandria	60	12	20	20	5	25	21.3
Sharkia	175	25	14.3	65	18	27.7	17.9
Behira	40	12	30	40	15	37.5	33.8
North Sinai	117	12	10.3	123	15	12.2	11.3
Qalioubia	140	45	32.1	20	6	30	31.9
Kafr El -Sheikh	125	47	37.6	35	15	42.9	38.8
Garbia	140	25	17.9	20	5	25	18.8
Dakahlia	85	31	36.5	75	28	37.3	36.9
Damietta	32	10	31.3	48	17	35.4	33.8
Menofia	62	16	25.8	18	5	27.8	26.3
Ismalia	60	12	20	20	5	25	21.3
Giza	60	10	16.7	20	5	25	18.8
Total	1096	257	23.8	504	139	27.6	25.1

¤ Overall percentage of sheep and goats for each governorate

Table 3: Number of samples showing CPE in relation to type of samples, species, age, sex and season

			Type of samples				Species			Age			Sex		Season	
Governorates	Farm	No of samples showing CPE	Nasal swabs	Vaginal swabs	Faecal swabs	Lung tissues	Local sheep	Foreign sheep	Local goat	0-1 Y(1)	1-2 Y	Over 2 Y	Male	Female	Summer	Winter
Kafr EL	EL Karada	13	11	2	-	-	-	13	-	2	5	6	5	8	11	2
Sheikh	Sakha	7	5	2	-	-	5	-	2	2	3	2	2	5	-	7
Giza	Oseam	1	1	-	-	-	1	-	-	-	-	1	1	-	1	-
	Private Farm	2	2	-	-	-	-	1	1	-	-	2	2	-	-	2
	Giza Abattoir	6	-	-	-	6	4	-	2	1	2	3	4	2	2	4
Alexanderia	Borg El- Arab	11	-	6	5	-	-	-	11	5	1	5	-	11	-	11
Total	-	40	19	10	5	6	10	14	16	10	11	19	14	26	14	26
Overall Percentage (2)	-	10.42	-	-	-	-	-	-	-	-	-	-	-	-	-	-

(1) Y = Year (2) Percentage in relation to total number of sample

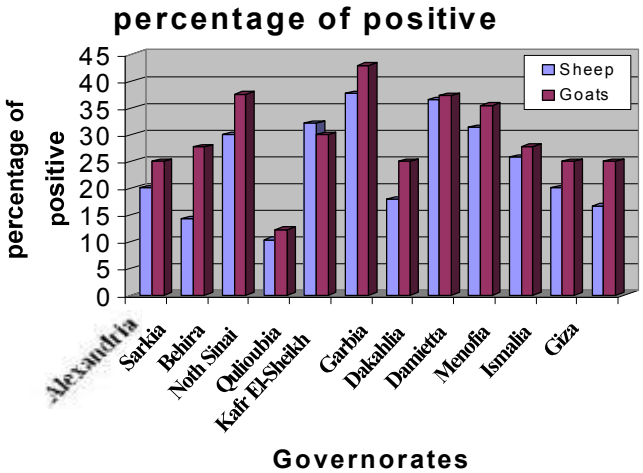

Fig. 1: Percentage of BoHV-1 specific antibodies among sheep and goats in 12 governorates in Lower Egypt

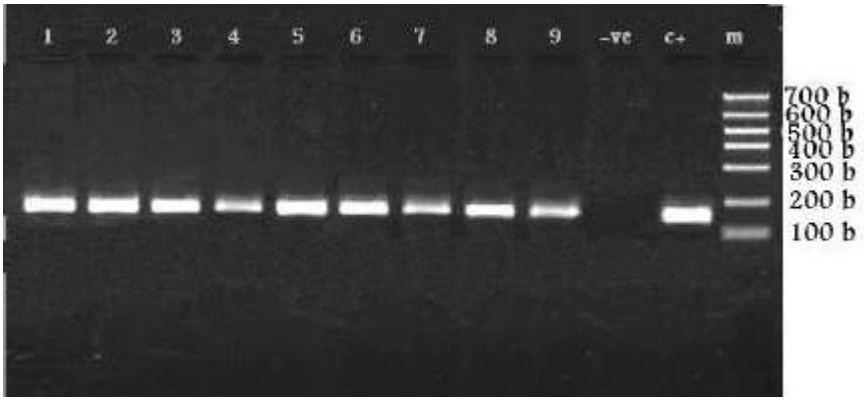

Fig. 2: The photo showed marker and results of the positive control strain of BoHV-1 and negative control, then followed by isolates No. 5,6,10,12,14,17,18, 25 and 26 of BoHV-1 in lane 1,2,3,4,5,6,7,8 and 9 respectively

Table 4: Results of virus neutralization test for identification of BoHV-1 isolates using standard BoHV-1 positive serum

Governorate	Farm	Number of isolates	Code number of isolates	Virus Neutralization Index (VNI) in Log 10
Kafr El Sheikh	El Karada	3	6	3.0
			10	2.9
			12	2.5
	Sakha	2	14	3.1
			17	1.6
Giza	Giza Abattoir	2	26	2.8
			29	1.6
Alexandria	Borg El- Arab	2	33	2.6
			36	2.2
Total	-	9	-	-

Table 5: Results of examination of the forty cytopathic viral agents by polymerase chain reaction

			Species			Type of samples			
Governorates	Farm	Code No.of positive samples	Local sheep	Foreign sheep	Local goats	Nasal swabs	Vaginal swabs	Faecal swabs	Lung tissues
Kafr El Sheikh	El Karada	5	-	+	-	+	-	-	-
		6	-	+	-	+	-	-	-
		10	-	+	-	+	-	-	-
		12	-	+	-	+	-	-	-
	Sakha	14	+	-	-	+	-	-	-
		17	-	-	+	-	+	-	-
		18	+	-	-	+	-	-	-
Giza	Giza Abattoir	25	-	-	+	-	-	-	+
		26	-	-	+	-	-	-	+
		29	+	-	-	-	-	-	+
Alexandria	Borg El- Arab	33	-	-	+	-	+	-	-
		36	-	-	+	-	+	-	-
Total	-	12	3	4	5	6	3	-	3

Table 6: Percentage of BoHV-1 isolates in relation to type of samples, species, age, sex, and season

			Type of samples				Species			Age			Sex		Season	
Governorates	Farm	No of samples showing CPE	Nasal swabs	Vaginal swabs	Faecal swabs	Lung tissues	Local sheep	Foreign sheep	Local goat	0-1 Y(1)	1-2 Y	Over 2 Y	Male	Female	Summer	Winter
Kafr EL	EL Karada	4	4	-	-	-	-	4	-	1	1	2	2	2	3	1
Sheikh	Sakha	3	2	1	-	-	2	-	1	1	1	1	1	2	-	3
Giza	Giza Abattoir	3	-	-	-	3	1	-	2	-	2	1	2	1	1	2
Alexanderia	Borg El Arab	2	-	2	-	-	-	-	2	-	-	2	-	2	-	2
Total	-	12	6	3	-	3	3	4	5	2	4	6	5	7	4	8
Overall Percentage	-	3.13 (2)	3.7 (3)	3.4	-	5.8	2.5	3.7	3.2	1.8	3.3	3.9	3.0	3.2	1.9	3.8

(1) Y = Year (2) Percentage in relation to total number of samples. (3) Percentage in relation to number of each type of samples and animals

Virus Isolation and Identification

Isolation of a Viral Agent from Different Swabs: The results of virus isolation from swabs are shown in Table 2 and Fig. 1. Out of 384 swabs only 40 (10.41%) showed cytopathic effect CPE on MDBK cells. Twenty cytopathic viral agents could be isolated from samples collected from Kafr El Sheikh, 9 from Giza and 11 from Alexanderia governorates, while samples collected from Qaloubia and Beheira were negative for virus isolation.

Virus Neutralization Test: Complete neutralization of the nine BoHV-1 isolates were occurred (no CPE) after 72 hours post inoculation of the virus - serum mixture on MDBK cells. Five BHV-1 isolates were obtained from Kafr El Sheik governorate, two from Giza governorate and two from Alexandria governorate as shown in Table 3.

Detection of BHV-1 DNA by Using Polymerase Chain Reaction: Twelve BoHV-1 isolates were detected by PCR, 7 BoHV-1 isolates from Kafr El Sheikh governorate, 3 BoHV-1 isolates from Giza governorate and 2 BHV-1 isolates from Alexandria governorate. Concerning the type of samples, 6 BoHV-1 isolates were detected from nasal swabs, 3 BoHV-1 isolates from vaginal swabs and 3 BoHV-1 isolates from lung tissues. No isolates could be detected from faecal swabs. Regarding to animal species, 5 BoHV-1 isolates were obtained from local goats, 4 BoHV-1 isolates from foreign sheep and 3 BoHV-1 isolates from local sheep (Fig. 2).

DISCUSSION

In the present work, screening of 1600 serum samples collected from sheep (1096) and goats (504) in 12 different Egyptian governorates by using blocking ELISA technique was carried out. This survey showed that the prevalence of BoHV-1 positive serum reactors among sheep and goats in Lower Egypt were 257 (23.4%), while the BoHV-1 positive serum reactors among goats were 139 (27.6%).

The highest percentages of positive BoHV-1 serum reactors were obtained from Kafr El-Sheikh, Dakahlia, Beheira, Damietta and Qalioubia governorates (38.8, 36.9, 33.8, 33.8 and 31.9%, respectively) and the lowest percentages of prevalence of BoHV-1 antibodies were recorded in North Sinai governorate (11.3%).

The comparison between the prevalence of BoHV-1 positive serum reactors revealed that the highest percentage of BHV-1 positive serum reactors were obtained in goats (27.6%) followed by sheep (23.8%). These results denoted that BoHV-1 are circulating among sheep and goats in examined governorates, so it is concluded that the virus is widely spread due to active infection rather than vaccination as there is no vaccination programme for sheep and goats. The recorded results are coincided with those reported in Greece [14] between 30 and 60% in southern Italy [15] and 21% in Spain [4].

The lowest rate of detection of specific antibodies against BoHV-1 presented in North Sinai governorate could be attributed to good climatic weather, management condition, animal distribution and low population of cattle. Moreover this governorate is separated from other governorates in Lower Egypt.

Variation in the rate of prevalence of BoHV-1 antibodies among sheep and goats in the tested governorates are depending on environmental condition, animal movement and the chance of exposure of these animals to BoHV-1 infection tacking in consideration the breeding of cattle, sheep and goats together under Egyptian field conditions which are the main factors that help the transmission of BoHV-1 infection from cattle to sheep and goats and vise versa.

IBR virus developed CEP forty eight hours post inoculation [16]. The characteristic CPE to IBR virus in the form of cell rounding and by progressing of the CPE, the cells formed a characteristic grape like appearance. Forty cytophathic inducing viral agents were isolated out of total 384 different samples, some of the isolates induced typical CPE characteristic for IBR virus 24-48 hours post inoculation such changes gave suspicion for the presence of BoHV-1 among certain isolates. The isolation was achieved from two farms at Kafr El Sheikh governorate, from two farms and Giza Abattoir at Giza governorate and from one farm at Alexandria governorate.

The forty BHV-1 isolates were examined by virus neutralization test by using control BHV-1 positive serum; nine out of them could be completely neutralized. Virus neutralization test is one of the conventional serological methods which are widely used for identification of BoHV-1 infection. A standard virus neutralization test was conducted [17] for confirmation of presence of BoHV-1 in the nine isolates and neutralization indexes were calculated. The neutralization index was different from one isolate to another with the same BoHV-1 positive serum and this may be attributed to the presence of minor antigenic differences between the isolated strains detectable by the virus neutralization test and the

difference in the titre of virus isolates, this results agreed with the finding of Kramps [18].

The PCR has the primary advantages of being more sensitive and more rapid; it could be performed in 1-2 days and could detect DNA in latently infected animals [12]. All forty cytopathic viral agents isolated from different samples were examined by PCR for detection of BHV-1 DNA using specific BoHV-1 primer and compared with positive and negative BHV-1 control. Twelve BHV-1 isolates could be detected by PCR .

The present results showed that PCR is more sensitive than VNT and this agreed with a group of authors such as [21-23]. The difference between PCR results and the VNT for detection of BHV-1 may be attributed to the titre of virus in the collected samples. The isolation and identification by conventional techniques requires at least 250 TCID50/ 0.05ml, while PCR requires 0.25-2.5 TCID50/ 0.05ml [21].

Lung tissues yielded the highest percentage of BoHV-1 isolates (5.8%), followed by nasal swabs (3.7%) and this may be due to overcrowdness and bad hygienic measures which play a role in the transmission of respiratory diseases in animal population.

The foreign sheep gave the highest percentage of BoHV-1 isolates (3.7%) followed by local goats (3.2%) and local sheep (2.5%) and this indicated that the 2 species are susceptible to infection [17]. Concerning the age of animals the highest percentage of BHV-1 isolates (3.9%) was found in animals over 2 years followed by animals between 1-2 years (3.3%) and this is considered a common age for animal breeding and also a suitable time for the transmission of infection from males to females and vice versa [24]. In addition the percentage of BoHV-1 isolates was found higher in winter (3.8%), while in summer (1.9%) and this may be attributed to high humidity and bad ventilation in animal houses in winter .

This investigation resulted in detection of 12 BoHV-1 isolates by PCR, 10 of them were isolated from clinically infected animals suffering from respiratory and genital disorders and two BoHV-1 isolates from apparently normal animals which have a latent infection, while 9 of the isolates were BoHV-1/IBR and 3 isolates were BoHV-1/IPV.

It could be concluded that BHV-1 is widely distributed among sheep and goats in Egypt. ELISA technique could be used for serosurvey as it is accurate, sensitive and specific for detection of BoHV-1 antibodies. PCR is the technique of choice for diagnosis of BoHV-1 as it is rapid, sensitive and more specific than conventional techniques.

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(Received: 08/07/2009; Accepted: 24/8/2009)