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Isolation and Identification of Some Viruses Causing Abortion in Equine in Egypt

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ABSTRACT

Equine Herpes Virus types 1 and 4 (EHV-1,4) and Equine Viral Arteritis (EVA) causes significant economic losses in Equine due to abortion and neonatal mortality. In this study, 80 samples were collected from pure Arabian horses suffering from abortion, respiratory illness and neurological problems, including nasal swabs, vaginal swabs and tissue samples (liver, lung, kidney, spleen, placenta of aborted fetuses and neonatal dead foal) from 2018 to 2021.All samples were inoculated on embryonated chicken eggs (ECE) via chorioallantoicmembrane (CAM).Indirect immunofluoresent antibody technique (IFAT) on CAM was used to serologically confirm the positive samples, Real Time and conventional PCR were used for further molecular confirmation . After 5-6 days of incubation, 22 out of 80 processed samples were positive in the form of pin-head necrotic foci on CAM.Yellowish green fluorescent granules were found in positive samples by IFAT. By Real Time PCR , 80 tissue samples tested were negative for EVA, but only six positive for EHVs (5 for EHV-4) and one for EHV-1, also by conventional PCR), with one of them testing positive for both EHV-1,4indicating natural co-infection incidence.Furthermore, eight casesout of 34 samples obtained in the spring season were EHVs-positive (26.67%), ten samples out of 21 collected in the fall were EHVs-positive (47.62%), and four samples out of 15 collected in the winter were EHVs-positive (25.53%). In conclusion, Abortion in Arabian horses in Egypt are mostly caused by EHVs especially in autumn while no evidence for EVA was detected in the collected samples.

Key words: Abortion, Arabian horses, ECE, EHV-(1, 4), EVA, IFAT and PCR.

INTRODUCTION

Viral causes of abortion (EHV-1, EHV-4 and EVA) causes significant economic losses in Equine, particularly Arabian horsesand causes a high rate of neonatal mortality. EHV-1 and EHV-4 are considered as the most common causative agents of abortion (Lunn et al., 2009). EHV-1, EHV-4 are linear double-stranded DNA viruses that belong to the *Herpesviridae* family, the genus *Varicellovirus* and the subfamily *Alphaherpesvirinae* (Davison, 2009). They are closely related alphaherpesviruses that cause significant

diseases in Equids (Gonzalez-Medina and Newton, 2015). Despite the fact that both EHV-1 and EHV-4 induce respiratory disease in Equine, only EHV-1 infection can cause abortion, perinatal death and neurological abnormalities with varying severity which can result in total paralysis (Patel & Heldens 2005). Although the viruses are reported to be relatively short-lived in the environment, direct contact with infected aborted foetuses, placental tissues or fomites contaminated by respiratory secretions may serve as a source of infection (Harless & Pusterla 2006). EHV-4 primarily infects epithelial cells, whereas EHV-1 also infects immune cells, these viruses are able to cause systemic infection inducing abortion, myeloencephalopathy and chorioretinopathy (Hussey et al., 2013). EHV-1, EHV-4 enter into a latent state in lymphoreticular system cells and trigeminal ganglia neurons (Slater et al., 1994), and reactivation from latency can result in recurring disease, virus shedding and transmission to other horses (Crabb & Studdert 1995). EHV-1 was isolated in Egypt from nasopharyngeal, trachea, lungs and liver samples of an aborted fetus by inoculation on embryonated chicken egg, It showed thickening or diffuse opacity of CAM with the presence of pin-head necrotic foci (Hassanain et al., 2002; Warda,2003; Soliman et al., 2008; AbdEl-Hafeiz et al. 2010). Furthermore, IFAT has been used to diagnose cases of equine abortion (OIE, 2013b). In Egypt, EHV-4 was detected using nested PCR and sequence analysis on fetus of Arabian horses (Al Shammari et al., 2016). Researchers could use semi-nested PCR to detect EHV-1, EHV-2, EHV-4 in clinical samples collected from Arabian horses between 2005 and 2006, using fetal tissues, nasal samples and uterine samples (Amer et al. 2011).

EVA is a virus that causes respiratory and reproductive problems in many Equine populations around the world (Timoney & McCollum, 1993). EAV is an enveloped virus with a single-stranded, positive-sense RNA genome of about 12.7 kb that belongs to the *Arteriviridae* family, *Nidovirales* order (Cavanagh, 1997). Although the vast majority of EAV infections are inapparent or subclinical, occurrence of outbreaks of EVA infection were recorded (Timoney & McCollum, 1993). Signs include influenza-like sickness in adult horses, abortion in pregnant mares and interstitial pneumonia in very young foals. The virus can spread in many different ways, the respiratory route is the most common. The virus can potentially spread by artificial insemination via the venereal route (McCollum et al., 1999).

The aim of the current study was to investigate the circulation of equid herpesviruses, particularly EHV-1 and EHV-4 and EVA in Arabian horses in Egypt.

MATERIAL AND METHODS

Between 2018 and 2021 in different seasons as indicated in Table (5), 80 samples were collected under aseptic conditions from pure Arabian horses suffering from abortion, respiratory infection and neurological disorders, including nasal swabs, vaginal swabs and tissue samples (liver, lung, kidney, spleen, placenta) of aborted fetuses and neonatal dead foal as shown in Table (1) which indicates the types and numbers of collected tissue samples. Specimens from fetal organs and placenta were transported directly to the Virology Department, Animal Health Research Institute (AHRI), El-Dokki, Giza, Egypt in a clean, dry and sterile ice box at 4°C with phosphate buffer saline (PBS) and stored at -70°C until use.

To make a 10% suspension, approximately 2 gm of collected aborted fetal tissues were mixed with an equal volume of phosphate buffer saline and ground in a sterile mortar with sterile sand. For 10 minutes, the tubes were centrifuged at 1500 rpm. The

supernatant fluid was collected in sterile screw-capped vials pH 7.2, 100IU/ml penicillin and 100µg/ml streptomycin were added and stored at – 70°C till use in virus isolation (OIE, 2013b).

Swabs were collected by inserting sterile cotton into the vaginal or nasal cavity to collect discharges from the inside cavity, then immediately soaking in 2 ml of transport media (maintenance media).The collected samples were kept on ice and transported to the laboratory as soon as possible. They were centrifuged for 10 minutes at 1500 rpm. The supernatants were collected in sterile screw-capped vials, labelled pH 7.2, 100IU/ml penicillin, and 100µg/ml streptomycin were added, and the vials were stored at -70°C till use in virus isolation (OIE, 2013b).

Table (1): Numbers and types of suspected field samples collected in Egypt:

Type of animal cases	Clinical manifestations	Types of sample	Number of collected samples
Mares	Abortion	Vaginal, Uterine swabs and tissues*	23
Neonatal foals	Still birth	Tissues*	10
Dead Fetus	Abortion	Tissues*	47
Total	-------	------	80

*Tissues (liver, lung, kidney, spleen, placenta) ofdead mares, aborted fetuses and neonatal dead foal

Virus isolation:

Specific pathogen free Embryonated Chickens Eggs (SPF-ECEs) were used for isolating viruses from all of the collected samples via CAM inoculation as recommended by (OIE, 2013b). Virus isolation is regarded as the gold standard test for EHV-1, EHV-4 (Lunn et al., 2009). The used SPF-ECEs were purchased from NIL SPF farm, komosheim- fayoum, Egypt.

The method: A 0.45µmmembrane filter was used to filter the supernatant of previously prepared tissue samples. A total of 150µl of each filtrated sample was inoculated on CAM of SPF-ECEs (11-13 days) and incubated at 37°C for 5 days, with twice daily examinations. SPF-ECEs died within 24 hours post inoculation were discarded and considered as Non-specific death.After three passages, the CAM were harvested aseptically and examined for changes in the CAM according to (OIE, 2013b).

Serological detection of EHV-1 in CAM using IFAT:

The CAM was harvested aseptically after three passages of EHV-1 samples, and a portion of some selected positive samples showed clear pock lesions was used for identification by IFAT using known Equine polyclonal antibodies against EHV-1, rabbit anti-equine conjugated with florescence isothiocyanate (FITC) obtained from (animal and plant health inspection services, national veterinary service laboratory, USA) .

The method: Freshly dissected CAM samples (5 ×5 mm) were cryo-sectioned at -30°C and mounted on microscope slides by cryostate machine. Cold acetone was used to fix cryo-sections for 30 minutes. After air drying, the sections were incubated with known Equine EHV-1polyclonal antibody for 30 minutes at 37°C in a humid environment. Three PBS washes were used to remove unreacted antibody. Anti-equine conjugated with florescence isothiocyanate (FITC) was added and incubated for 30 minutesbefore washing. The tissue sections were then covered with a cover slip and

aqueous mounting buffer (1 glycerin: 9 PBS).The slides were examined using a fluorescent microscope at 40x magnificationas described by Payment and Trudel (1993).

Molecular diagnosis:

Detection of EVA byQuantitative Real-Time PCR (qRT-PCR):

This approach can be used to identify EVA in a variety of samples, including nasal swabs, vaginal swabs and tissue samples of aborted fetuses and dead mares. Kogene biotech's RNA extraction kit was used to extract RNA (REF E007).Master Mix qRT-PCR Primer design (Oasig^{TM} lyophilized) one step 2x was used.The primer and probe mix provided exploits, the so-called TaqMan® principle. During PCR amplification, forward and reverse primers hybridize to the EAV cDNA. A fluorogenic probe is included in the same reaction mixture which consists of a DNA probe labeled with a 5`-dye and a 3`-quencher. During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated. The resulting increase in fluorescence can be detected on a range of qPCR platforms .Initial denaturation at 42°C for 10 minutes and 95°C for 2 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The obtained results are recorded on a form (20.1). Positive samples are expected to amplify between Cq 16 and 23 (OIE, 2013).

Detection of EHVs by Real Time and Conventional PCR (qPCR):

Firstly, Real time PCR was used to identify EHVs in the prepared tissue samples (placenta, liver, lung and spleen of aborted fetuses and dead mares), as well as nasal/vaginal swabs of mares, using the G-spin^{TM}DNA Extraction kit, according to manufacture instructions (REF 17045). Master Mix qRT-PCR Primer design (Oasig^{TM} lyophilized) one step 2x was used. A thermocycler was used, the first step of activation was performed at 95°C for 2 minutes. After that, 40 cycles of denaturation at 95°C for 10 seconds were performed, 1 minute of hybridization/extension and data collection at 60°C .The obtained results are recorded on a form (20.1) (OIE, 2018).

By using Conventional PCR (qPCR) for detection of EHV-1 of some selected samples, selective amplification of specified targeted of DNA sequence in gB gene for EHV-1 (342bp) Table (2) (Hafshejani et al., 2015). Extraction of DNA of EHV-1 using the QIAamp DNA Mini Kit according to manufacturer's instructions.PCR reactions were performed usingEmerald Amp GT PCR mastermix (Takara) kit, forward and reverse primers Table (2). A thermocycler was used,primary denaturation at 95°C for 5 minutes, secondary denaturation at 94°C for 30 sec, annealing at 55˚C for 40 sec, extension at 72˚C for 40 sec and final extension 72˚C for 10 minutes after 35 cycles Table (3).

Table (2): Oligonucleotide primers sequences of EHV-1by conventional PCR [Source: Midland Certified Reagent Companyoilgos (USA)]:

Gene	Primer Sequence 5'-3'	Amplified product	Reference
EHV-1 *glycoprotein B*	GCAAACAACAGAGGGTCGATAGAAG GTCGATGTCGTAAAACCTGAGAG	342bp	**Hafshejani*et al.*, 2015**

Table (3): Cycling conditions of the different primers during cPCR according to Emerald Amp GT PCR mastermix (Takara) kit:

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
EH1 glycoprotein B	95˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 40 sec.	35	72˚C 10 min.

RESULTS

Virus Isolation in Embryonated Chicken Eggs:

After 5-6 days of incubation after three passages, only 22 out of 80 samples were positive on CAM of ECE in the form of thickening or diffuse opacity of CAM with the presence of some pin-head necrotic foci, Fig (2-4). 58 negative CAM were shown in Fig. (1)

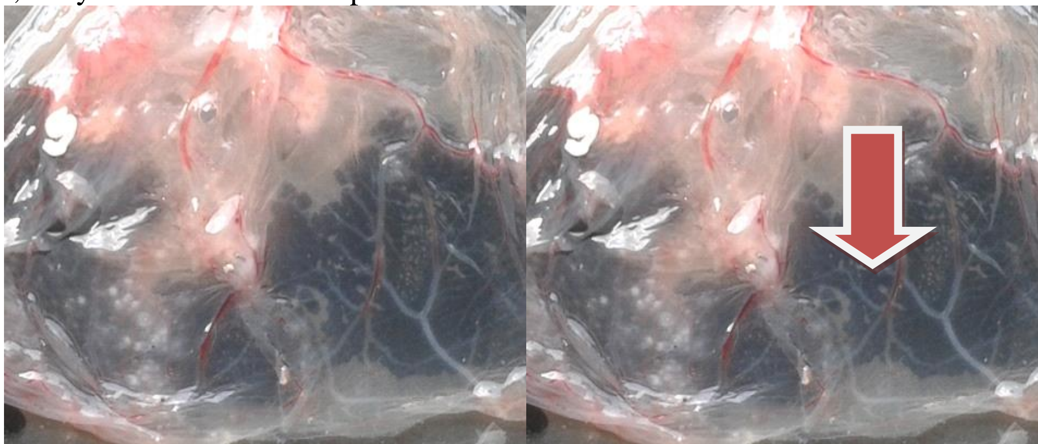


Fig. (1) Negative CAM.

Fig. (2) Positive CAM showing Pin headed necrotic foci and hemorrhage.

Fig.(3) Positive CAM showing dark redpatches on fetal membrane

Fig.(4) Positive CAM showing vasculitis and organized thrombus within blood vessel.

Identification of positive isolates by IFAT:

Selected positive samples expressive pocks on CAM examined by IFAT showed (the presence of yellowish green fluorescent granules which indicating the presence of viral antigen on pock lesions) using specific EHV-1 antisera Fig. (6)

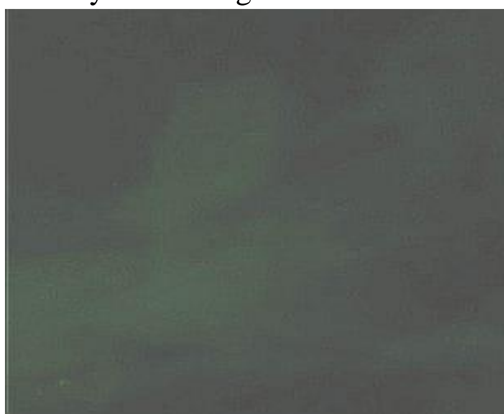


Fig. (5) Negative control

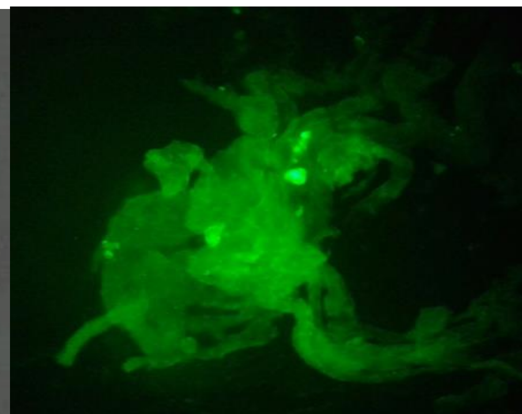


Fig. (6): Showing the presence of yellowish green fluorescent granules which indicating the presence of viral antigen on pock lesions

Real Time PCR for diagnosis of EHV-1, EHV-4 and EVA virus and conventional PCR for EHV-1:

80 nasal, vaginal of mares and tissue samples of dead mares, aborted fetuses and neonatal dead foals were negative for EVA virus by Real Time PCR.

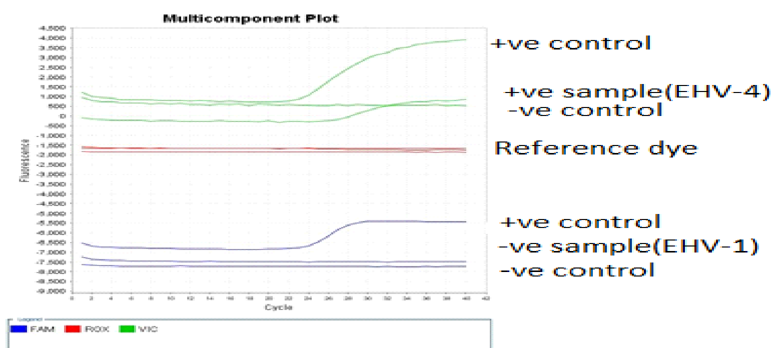


Fig. (7) Only 5 samples out of 80 samples were positive for EHV- 4 by real time PCR and were shown in and Ct values were shown in Table (4).

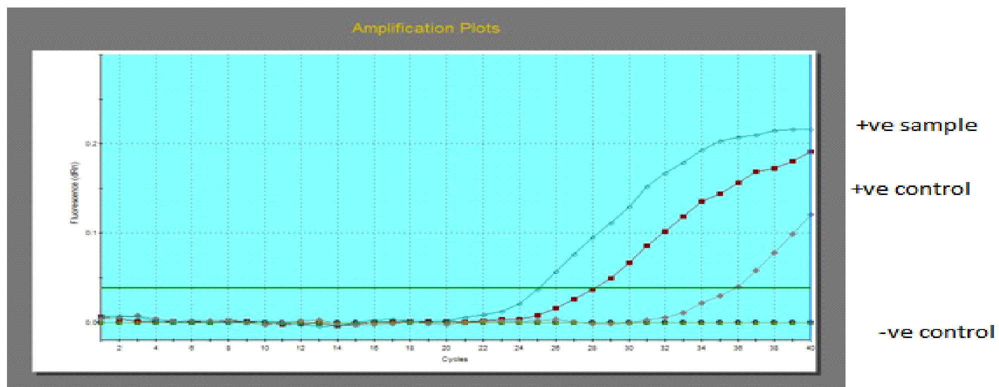
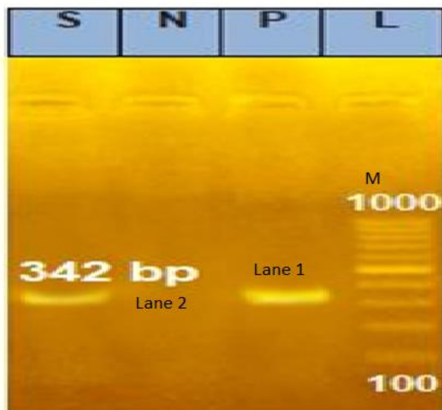


Fig.(8) One Sample was positive for EHV-1 by Real Time PCR with CT value (32)



Lane 1 : Positive control for EHV-1
Lane2 : Negative control for EHV-1
Lane3 :Positive sample for EHV-1(directly tested by PCR)

Fig.(9): Detection of EHV-1 for some selected clinical samples using Uniplex PCR assays; Show M: DNA ladder (marker) , +ve and – ve control and the predicted molecular size of DNA band at 342bp at the second PCR round of g B gene , at lane 3 show + ve sample.

Table (4): Results of Real time PCR for diagnosis of EHV-1,4 and EVA and CAM results:

Clinical data	**Sample type**	**Result of Real time PCR**			**CT**	**CAM**
		EHV-1	**EHV-4**	**EAV**		
Aborted fetus	Tissue samples - swabs	+	+	-	32	+

Aborted fetus	Tissue samples	-	+	-	34	+
Recently aborted mare	Tissue samples	-	+	-	34	+
Recently aborted mare	Tissue samples	-	+	-	32	+
Aborted fetus	Tissue samples	-	+	-	36	+

Statistical analysis by ANOVA:

During the period of 2018 to 2021, there were 80 incidences of abortion from pregnant mares and neonatal deaths in Egypt. Out of 34 samples, 8 cases were positive (26.67%) during the spring, out of 21 samples, 10 were positive (47.62%) during the autumn, and out of 15 samples 4 were positive (25.53%) during the winter, indicating a high incidence of abortion throughout the autumn, winter, spring and followed by a reduction in the summer as was shown in Table (5) and Fig. (10).

Table (5): History of aborted cases and neonatal deaths:

Season	Spring	Autumn	Winter	Summer
No. positive samlpes/Total No.	8/34	10/21	4/15	0/10
Collection date	April 2018	September 2019	January 2020	June 2021
Age of dam	5-9 Y	5-9Y	5-9Y	5-9Y
Vaccination against EHV-1, EHV-4	(Inactivated)	(Inactivated)	(Inactivated)	(Inactivated)

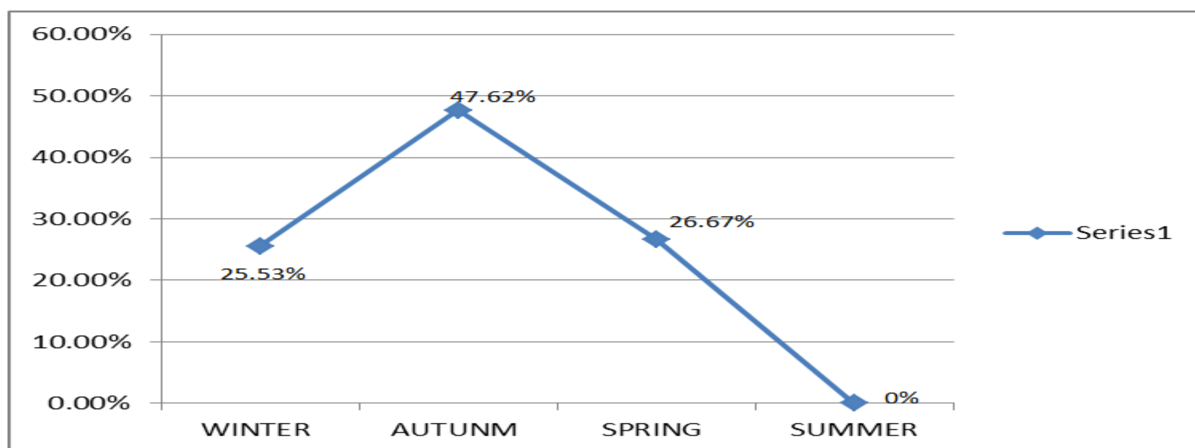


Figure (10): Showing the results of statistical analysis of the most seasons of abortion.

Obtained data was analyzed as one way ANOVA, using Proc GLM in SAS (Anonymous. 2003). Means were compared by Duncan multiple range test (*P*= 0.05 level) in the same program.

DISCUSSION

Abortion causes significant economic losses in equines, particularly Arabian horses, and causes a high rate of neonatal mortality (Lunn et al., 2009). EHV-1, 4 and EVA are viral causes of abortion. According to our findings, EHV-1, 4 is one of the most common causes of abortion in horses, is highly contagious and is usually transmitted through direct contact primarily through infected aborted fetuses, placental tissues or fomites contaminated by respiratory secretions (Harless & Pusterla 2006). The establishment of lifelong latency in a large proportion of infected animals ensures herpesvirus survival in equine populations and allows the virus to be shed sporadically throughout the host's lifetime (Gonzalez-Medina and Newton, 2015).

In our study, we used various diagnostic techniques to confirm the diagnosis of viruses causing abortion in the equine population. Virus isolation is regarded as the gold standard test for EHV-1, EHV-4 (Lunn et al., 2009). So, ECE 11-13 days old were used to isolate EHVs from clinical samples via CAM inoculation. The detection rate of twenty-two positive samples from eighty samples (27.5%) showing CPE in the form of thickening or diffuse opacity of CAM with the presence of some pin-head necrotic foci and dark red patches on fetal membrane, vasculitis, and organised thrombus within blood vessel Fig. (2, 3, 4), this was relatively low when compared to previous studies that found 41.66 percent (El sayyad et al., 2015). These results were in agreement with that obtained by (Hassanain et al., 2002, Warda, 2003) who isolated EHV-1 from aborted fetalorgans on CAM of embryonated chicken eggs.

IFAT is highly recommended for Equine herpes virus diagnosis and identification from tissues of aborted fetusesand strongly supportiveofvirusdetection. Furthermore, the IFAT technique has been compared to viral isolation using cell cultures in more than 100 cases of equine abortion showing that both techniques are similar in terms of diagnostic reliability (OIE, 2013b). So, we used IFAT for identification and confirmation of selected positive samples using known Equine polyclonal antibodies against EHV-1, the result confirmed the presence of yellowish green florescent granules in tested pock lesions (Fig. 5). The

results of IFAT in this study give agreement with that obtained by (Abdel-Hafez et al.2010) who isolated EHV-1from fetal fluids of aborted foreign mares at 5-7th months of gestation in horse having suggestive EHV-1 infection.

The various PCR techniques are rapid diagnostic tests used to detect EHV-1, EHV-4 and EVA at the molecular level. The diagnosis by PCR was used to determine viral load in tissue samples from aborted equines for EHV-1, EHV-4 *(*OIE, 2013d). In Egypt, the epidemiological picture of EVA is still unclear, with no data on the infection available. As a result, in the current study,Real Time PCR was performedon nasal, vaginal swabs of mares and tissue samples of dead mares, aborted fetuses and neonatal dead foals for detection of EVA, but all samples were negative, despite the fact that the World Organization for Animal Health (OIE) detected EVA in the UK, Mainland and Europe between 1997 and 2018. The virus is widely distributed around the world (Timoney and McCollum, 1993), but the clinical and subclinical prevalence varies by country and breed. The most extensive recorded occurrence of EVA in Quarter Horses in the United States was caused primarily by shipment of virus-infective sperm and movement of donor or embryo recipient mares and was then isolated from the placenta of an aborted fetus (Powell &Timoney, 2006; Timoney et al., 2006). EVA was found in semen and tissue samples, as well as the development and evaluation of a reverse transcription-insulated iso thermal PCR assay for detection of EVA infection (Carossino et al., 2016).

Furthermore , Real Time PCR was applied on nasal, vaginal swabs of mares and tissue samples of dead mares,aborted fetuses and neonatal dead foals, yielding positive results of five samples for EHV-4 (Fig. 8) and positive result of one sample for EHV-1with one of them testing positive for both EHV-1 and EHV-4 indicating natural co-infection incidence, also we used conventional PCR for detection of EHV-1 only one sample was positive (Fig. 9) indicating that EHV-4 is more detected than EHV-1. Our findings agreed with those of Elia et al. (2006), Abdel-Hafez et al. (2010) & Amer et al. (2011) who detected EHV-1 and EHV-4 by PCR in several infected equine fetal tissues.

In our study, we performed statistical analysis by ANOVA on 34 samples collected in the spring (eight cases were positive (26.67%), 21 samples collected in the autumn (ten were positive (47.62 %), and 15 samples collected in the winter (four were positive (25.53%), indicating that the incidence of abortion is highest in the autumn, winter and spring and decreases in the summer Table (5), Fig. (10). These findings agreed with those of Emad et al. (2018) who found that multiple risk factors, including season, fall and spring seasons have a significant impact on the prevalence of EHV-1 infection.

CONCLUSION

Due to the latency of EHV-1 and EHV-4 with the stress factor and high incidence of abortion in autumn, winter and spring indicating the importance of these viruses and worldwide spread causing a lot of economic losses.

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