Comparative Study between ELISA results and rRT-PCR results for Equine Arteritis virus in Thoroughbred and Foreign Horse Breeds in Egypt

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ABSTRACT

Till now the incidence of equine arteritis virus in Egypt is still unknown and need more investigations.This initiated the present study to design a protocol for isolation trials and characterization of equine arteritis virus from field specimens. A total of 548 samples were collected from governmental and private studs of Arabian horses and also foreign horse breeds, including 540 serum samples, among these, 4 EDTA-blood and 4 semen samples. Serologically, indirect ELISA was performed on 540 serum samples. 130 samples were clearly strong positive at a percentage of 24%. Molecular detection of EAV genome was performed only on 8 selected semen and EDTA-blood samples which were giving highly distinct positive reaction in the indirect ELISA. All samples were found negative to the presence of EAV genome by real time RT-PCR. Trials of isolation on different types of cell cultures were done on the 4 described semen samples; RK-13, VERO-1008, BHK-21 AND MDBK cell lines were used in these trials. Six blind serial passages were done using the described samples; all samples were found negative for the presence of CPE characteristic to EAV. All sixpassages of all samples on RK-13 cells were re-tested using rRT-PCR to confirm absence of EAV, thus assuring the absence of EAV in Egypt or at least in areas from which clinical specimens were collected..

The aim of this workto stand on the fact of presence or not of EAV in Egypt to either start a vaccination program or eliminate it from Egypt equine epidemics.

INTRODUCTION

Equine viral arteritis (EVA) is a contagious viral disease of equids caused by equine arteritis virus (EAV), a positive-sense, single-stranded RNA virus, and the prototype member of the genus *Arterivirus*, family *Arteriviridae*, order *Nidovirales* (CAVANAGH D.1997). EAV is present in the horse population of many countries world-wide (TIMONEY P.J. and MCCOLLUM W.H.,1993). There has been an increase in the incidence of EVA in recent years that has been linked to the greater frequency of movement of horses and use of transported semen (TIMONEY P.J. and MCCOLLUM W.H.1993 ;BALASURIYA U.B.R *et al*.,1998).

While the majority of cases of acute infection with EAV are subclinical, certain strains of the virus can cause disease of varying severity (TIMONEY P.J. and MCCOLLUM W.H.,1993). Typical cases of EVA can present with all or any combination of the following clinical signs: fever, depression, anorexia, leucopenia, dependent edema, especially of the limbs, scrotum and prepuce of the stallion, conjunctivitis, ocular discharge, supra or periorbital edema, rhinitis, nasal discharge, a local or

generalized urticarial skin reaction, abortion, stillbirths and, rarely, a fulminating pneumonia, enteritis or pneumo-enteritis in young foals. Regardless of the severity of clinical signs, affected horses almost invariably make complete recoveries. The case-fatality rate in outbreaks of EVA is very low; mortality is usually only seen in very young foals, particularly those congenitally infected with the virus (VAALA W.E *et al*.,1992;TIMONEY P.J. and MCCOLLUM W.H.,1993;LOPEZ J.W *et al*.,1996), and very rarely in otherwise healthy adult horses.

The disease which is present in horse populations in many countries is found in a wide range of breeds, more in some than in others. For example, in Arabians and Thoroughbreds it may occur in only one to three percent of the population, whereas it may reach 70 to 80 percent in Standard breeds. What makes the disease so important is that certain strains of EAV can cause abortion in susceptible mares and a significant percentage of stallions may become carriers. Even more importantly, it appears that persistent infection of stallions with EAV is more widespread among the various breeds than previously thought. This has significant economic implications for the commercial breeding industry, as carrier stallions can transmit EAV very efficiently, either through natural breeding, as is required in the Thoroughbred industry, or through artificial insemination, approved by virtually every other breed.

MATERIALS & METHODS

Samples: In this study a total of 548 samples were collected from adult and young Arabian horses, local horses during the period starting from February 2012 until June 2013. the samples included 540 serum samples; 4 EDTA- Blood samples and 4 semen samples.The samples were collected from all governorates of Egypt in addition to all horse clubs; all governmental agencies that use horses like police stations in some governorates, mounted police; army studs like army second battalion ; governmental studs like AL-Zahraa stud for Arabian horses and also from most famous private studs in all governorates. Serial decimal dilutions (10^{-1}–10^{-3}) of seminal plasma were done in tissue culture maintenance medium containing 2% fetal bovine serum and antibiotics.

Note: To avoid co-purification of cellular DNA, the use of cell-free body fluids for preparation of viral RNA is recommended so whole semen samples were centrifuged for 10 minutes at 1500 x g and the supernatant was used.

Detection of EAV antibodies in serum samples using indirect ELISA: The test is carried out by(INGEZIM ARTERITIS 14.EA.K1) ELISA kit subsidiary of (INGENASA) company.

The test method is described in details in kit enclosed pamphlet.

Detection of EAV RNA in Semen and EDTA-Blood samples from ELISA-positive Horses using rRT-PCR assay:

Total RNA extraction:

The test is carried out by QIAamp Viral RNA Kit (QIAGEN)

Detection of EAV RNA using rRT-PCR assay:

The test is carried out by Taq Vet Equine Arteritis Virus Kit (Lsi) using the 1 tube (1x) ready to use Master Mix (Mix EAV) which contain:

1) 1 Set of Nucleotides EAV:

- 1 Forward primer.
- 1 Reverse primer.

- 1 Probe EAV - TaqMan probe labeled in FAM-MGB (Quencher none).

2) 1 Set of nucleotides IPC

- 1 Forward primer.
- 1 Reverse primer.
- 1 Probe IPC - probe TaqMan labeled in VIC-MGB (Quencher none).

3) The Master Mix RT-PCR.
4) The specific enzyme RT-PCR.
5) 1 Tube of EPC (External positive control - EPC-EAV).
6) 1 Tube of exogenous IPC (internal positive control).

Comparative trials for isolation of EAV from semen samples in different types of cell culture:
cell lines:

- RK-13 (ATCC-CCL-37) Cell line is the cell system of choice (TIMONEY P.J. *et al*.,2004) for isolation of EAVit was obtained from cell culture department, VACSERA.
- BHK-21 Baby Hamster Kidney-21; (ATCC-CCL-10)
- Vero-1008(African green monkey kidney);(ATCC-CCL-81)
- MDBK (NBL-1) (Median derby bovine kidney);(ATCC-CCL-22)

They are obtained from C.C unit of virology department, animal health research institute

- Comparative trials for EAV isolation are attempted in 4 different types of C.C; RK-13, VERO, MDBK and BHK.

Method:

1) After removal of culture medium, 3–5-day-old confluent monolayer cultures of RK-13 cells, VERO, BHK and MDBK cell lines in 96-well tissue culture plates, were inoculated with serial decimal dilutions (10^{-1}–10^{-3}) of the previously prepared 4 semen samples separately.
2) The lids replaced on the plates and inoculated cultures gently rotated to disperse the inoculums over the cells monolayer.
3) Inoculated cultures are then incubated for 1 hour at 37°C either in an incubator containing a humidified atmosphere of 5% CO_2 in air.
4) Without removing any of the inoculums or washing the cells monolayer, the latter were overlaid with 0.75% carboxymethyl cellulose containing medium with antibiotics.
5) The plates were re-incubated at 37°C and checked microscopically for viral CPE, which is usually evident within 2–6 days.
6) In the absence of visible CPE, culture supernatants are sub-inoculated as a second passage onto 3–5 day-old confluent cell monolayer cultures of the described types of cells after 5 times of repeated freezing and thawing cycles for the first passage plates to guarantee rupture of cells containing target virus.
7) The last step was repeated in all types of the described cells up to the 6^{th} passage.

The supernatant of samples 6^{th} passage in the most sensitive cell line for EVA; RK-13 was collected separately to re-run rRT-PCR for molecular confirmation.

RESULTS

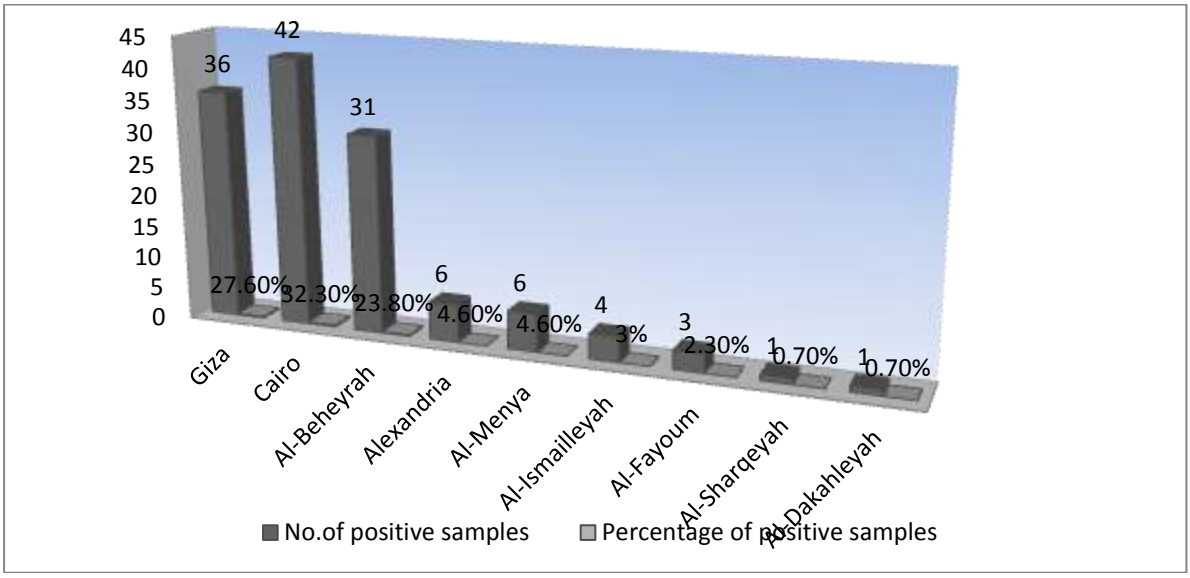
Results of indirect ELISA for detection of EAV antibodies in collected serum samples: The reaction of different samples in indirect ELISA was monitored using automated ELISA reader at OD 450 nm according

to the kit instructions and compared with positive and negative controls.

The number of positive samples in indirect ELISA is accordingly only 130 serum samples from 540 samples.

Governorate	No. of positive samples	percentage
Giza	**36**	**27.6 %**
Cairo	**42**	**32.3 %**
Al-Behirah	**31**	**23.8 %**
Alexandria	**6**	**4.6 %**
Al-Menya	**6**	**4.6 %**
Al-Ismailleyah	**4**	**3 %**
Al-Fayoum	**3**	**2.3 %**
Al-Sharqeyah	**1**	**0.7 %**
Al-Dakahleyah	**1**	**0.7 %**

No. of positive samples and its percentage in different governorates

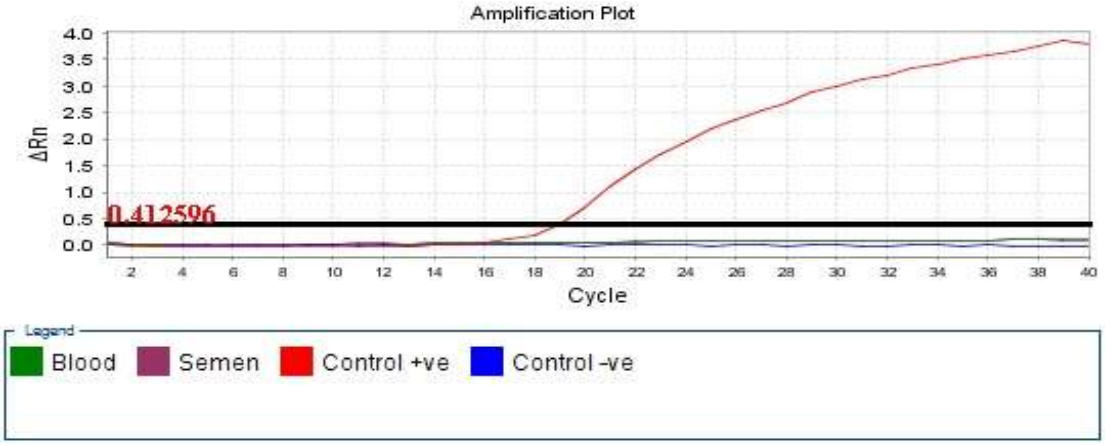


A figure representing the no. and percentage of positive samples to EAV antibodies in different governorates.

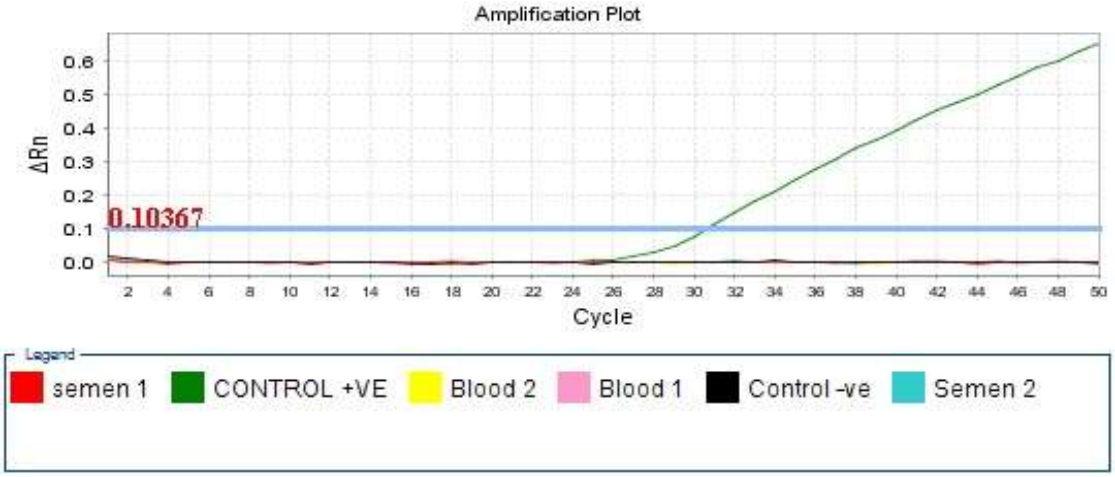
Results of rRT-PCR for detection of EAV RNA in collected samples:4 EDTA-Blood samples and 4 semen samples were selected from animals showed distinct positive reaction when subjected to indirect ELISA indicating high antibodies titer to EAV, were subjected to rRT-PCR for detection of EAV.

All samples were found Negative to the presence of EAV genome

rRT-PCR Results of 1st EDTA-Blood and 1st Semen samples.



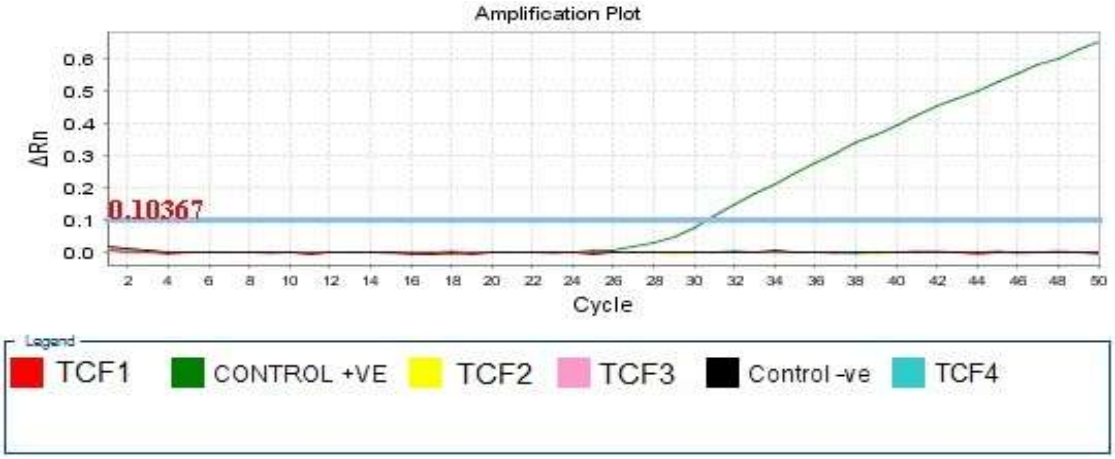
rRT-PCR Results of 2nd EDTA-Blood and 2nd semen samples.



rRT-PCR results of 3rd and 4th EDTA-Blood and 3rd and 4th semen samples.

Results of isolation trials of EAV on different types of cell cultures:since rRT-PCR results were negative, we had to make isolation trials on the described specific cell lines to make either virus isolation shown by specific CPE and confirmed later or virus amplification to re-run rRT-PCR to confirm presence or not of EAV.six blind serial passages were done using the described samples and cell lines. No CPE was detected on any type of this cell lines.

Results of rRT-PCR in TCF of RK-13 C.C: The 6th passage of the most sensitive cell line for EAV; RK-13 was chosen in all samples to re-test with rRT-PCR after supposed virus amplification; result was also negative in all samples for the presence of EAV genome.



rRT-PCR results of TCF of the 6th passage of 4 semen samples in RK-13 cell line.

Discussion

The first approach for detection of equine arteritis virus was by using indirect ELISA for detection of equine arteritis viral antibodies based on the possibility of the presence of EAV antibodies in serum due to past infection with EAV **(CHIRNSIDE E.D. *et al*., 1995;HEDGES J.F. *et al*.,1998;INIGUEZ P. *et al*.,1998;KONDO T. *et al*.,1998;CHO H.J. *et al*.,2000;NUGENT J.*et al*.,2000).**this is may be due to :

1-presence of EAV antibodies in tested horses serum due to past infection with the mentioned virus.

2-Due to importation of Arabian thoroughbred previously vaccinated with EAV vaccine in their home country.

3-vaccination of the existing Arabian thoroughbred with imported vaccine to EAV either single or combined with other equine vaccines.

4-false positive reaction associated with the presence of antibodies to various tissue culture antigens in the sera of horses that had been vaccinated with tissue-culture-derived vaccines.

So we moved to next step, rRT-PCR for detection of EAV genome in the described samples. The one tube TaqMan® rRT-PCR assays have been developed and evaluated for the detection of various strains of EAV in tissue culture fluid, semen and nasal secretions **(ST-LAURENT G. *et al*.,1994;GILBERT S.A. *et al*.,1997;STARICK E.,1998;RAMINA A. *et al*.,1999; SEKIGUCHI K. *et al*.,2000;BALASURIYA U.B.R. *et al*.,2002; WESTCOTT D.G. *et al*.,2003;BALASURIYA U.B.R. *et al*.,2004;ZHANG J. *et*

***al*.,2004;SZEREDI L. *et al*.,2005; LU Z *et al*., 2007).**
According to the OIE, 2 TaqMan® fluorogenic probe-based one-tube rRT-PCR assays have been described for the detection of EAV nucleic acid (**BALASURIYA U.B.R.*et al*.,2002**); primers ([forward: 5'-GGC-GAC-AGCCTA-CAA-GCT-ACA-3', reverse: 5'-CGG-CAT-CTG-CAG-TGA-GTG-A-3'] and probe [5'FAM-TTG-CGGACC-CGC-ATC-TGA-CCA-A-TAMRA-3'] and (**WESTCOTT D.G.*et al*.,2003**); primers [forward: 5'-GTA-CAC-CGC-AGT-TGG-TAA-CA-3', reverse: 5'-ACT-TCA-ACA-TGA-CGC-CAC-AC-3'] and probe [5'FAM-TGG-TTC-ACT-CAC-TGC-AGATGC-CGG-TAMRA-3']).In our research the second TaqMan® fluorogenic probe-based one-tube rRT-PCR assay is used with the same primers and probe.In this test all samples were found negative for the presence of EAV genome which were collected from Sero-positive stallions, and here should be noted that infected stallions remains long-life virus carriers and semen shedders **(Timoney P.J. *et al*.,1987**)., this result may be due to:
1-Absence of the virus from the beginning in the tested samples.
2-Genomic variation among field isolates of EAV could reduce the sensitivity of both RT-PCR and rRT-PCR assays, even when the primers and probe are based on the most conserved region of the EAV genome (ORF 7 [**LU Z.*et al*.,2007**]).
3-The EAV titer not enough to give positive results with rRT-PCR which has whatever a limited sensitivity.
So we moved to the last station in EAV diagnosis in the current study; isolation trials on the described 4 semen samples from stallions considered to be possible carriers **(TIMONEY P.J. & MCCOLLUM W.H. (1993)** on different types of specific cell lines. six serial passages with serial decimal dilutions were done (as described in OIE manual) on the described cell lines for either virus isolation or amplifying the virus titer to re-run rRT-PCR but also the result was negative for presence of CPE for EAV. But as The identity of isolates of EAV can be confirmed by rRT-PCR assay **(CHIRNSIDE E.D. & SPAAN W.J. 1990; ST-LAURENT G.et al.,1993; BALASURIYA U.B.R.et al.,1998)**; so we re-tested 4 TCF of the 6th passage of the inoculated samples in the most sensitive cell line for EAV; RK-13 cells by rRT-PCR to confirm the results and end the doubt.
The result was also negative for the presence of EAV genome in the tested samples.

In conclusion:This study is the first study on EAV in Egypt, it was necessary to investigate such important virus to exclude it from existing causative equine epidemics.
The present study assured absence of EAV at least from collecting areas of clinical specimens used.

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