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RESEARCH

First Isolation and identification of EHV-4 during abortion outbreak among Arabian horses in Egypt reflects an alteration in pathogenic potentiality of EHV-4

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ABSTRACT

Background: Usually EHV-4 was strictly confined to upper respiratory tract infection with rare incidence of abortion due to rare occurrence of cell associated Viraemia unlike EHV-1 infection, however; suspected herpes viral abortion cases in that outbreak were diagnosed and confirmed EHV-4 cases, Being considered an alteration in pathogenic potentiality of the virus.
Objective: to possess an EHV-4 full identified isolate from field strains to be used as a seed virus in diagnostic purposes later and to update the national monovalent vaccine of EHV-1.
Methods: Full molecular identified two EHV-4 PCR positive samples with a GenBank accession numbers of: KP699582, KX866264 were used in the current study. The samples were obtained during an outbreak of abortion in Arabian horse stud in Egypt during foaling season of 2014-2015. Isolation was carried out on MDBK cell line, identification of EHV-4 isolate was performed using IFAT, AGID and nPCR.
Results: Specific CPE was observed between 4-6th days in first and second passages, time was shortened till development of complete CPE till 2nd day in 5th and 6th passages. EHV-4 isolate was identified by detection of viral antigen with indirect IFAT, AGID tests using reference antisera and finally by nested PCR. The isolate was titrated on MDBK Cell culture revealing a titer of 106.75
Conclusion: EHV-4 can be detected and isolated from abortion cases, also to have an EHV-4 isolate is the first step to follow up the situation of EHV-4 in Egypt as a preliminary step for control of the disease.

Key words: Isolation, MDBK, IFAT, AGID, EHV-4

BACKGROUND

EHVs are ubiquitous herpes viruses that infect equine population worldwide. The latest taxonomy classifies Herpes Viruses within a new order, Herpesvirales, which is divided into three families: Herpesviridae, Alloherpesviridae, and Malacoher-pesviridae, In Equids 9 EHVs are identified so far (Davison *et al*., 2009), the most important of them are EHV-1, 4, Due to high capabilities to produce a serious problems among equine population. EHVs are DNA viruses of an asymmetrical outer envelope approximately 200 mμ in diameter, within which there is an icosahedral capsid, of about 100 mμ in diameter (O'Callaghan *et al*., 1983) with Linear double stranded, non-segmented genomes of length of 144 – 150 kbp (Darlington and Randall, 1963; Plummer and Waterson, 1963). EHV-1, 4 are highly contagious among susceptible horses, with viral transmission to cohort animals occurring by inhalation of aerosols of virus-laden respiratory secretions. A silent feature of herpes viruses' biology is their ability to produce a latent infection that persists for the lifetime of the host which complicating designs for control of these viruses (Foote *et al*., 2006), also EHVs are considered masters of immune evasion (van der Meulen *et al*., 2006); The two features which make control of the disease is very difficult. EHV-1, 4 have a nucleotide sequence identity ranges from 55-84% with subsequently high antigenic similarity; however, they are strikingly different regarding their pathogenesis, while EHV-4 is limited to the upper respiratory tract, EHV-1 is systemic distributed among different organs causing multiple disease forms, ranging from mild URT infection, rhinopneumonitis, to abortion storms, neonatal foal deaths and lethal Myeloencephalopathy, Both EHV-1 and EHV-4 can cause respiratory

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disease with variable severity (Patel and Heldens, 2005). But the difference being in after respiratory infection, while EHV-1 invades the lamina propria using migrating mononuclear cells as a vehicles for further distribution, As a result, subsequently spreads throughout the body via a cell-associated Viremia, reaching the secondary sites of virus replication, mainly the vasculature of the pregnant uterus and/or the central nervous system (CNS) to become responsible for its pathogenic picture. (Osterrieder and Van de Walle, 2010). It seems likely that leukocyte-associated Viremia and the subsequent infection of endothelial cells are key prerequisites for EHV-1 pathogenicity unlike to EHV-4 infection which is usually remains limited to the URT and rarely accompanied by cell-associated Viremia. Consequently; there is a difference between both mentioned strains in host range of cell culture based on difference in their capabilities of cell associated Viremia (Burrows and Goodridge 1973 and Allen and Brayen 1986), in consistence with the above mentioned; EHV-4 was shown to grow on wider range of cells unlike EHV-1, like RK-13, equine embryonic lung kidney (Taouji *et al*., 2002); in conflict with isolation trials during comparative study during 2006, EHV-4 was grown only on equine dermal cells (ED) (Diallo *et al*, 2006). It seems that EHV-4 somewhat restricted to cells of equine, rabbit or Feline origin (O'Callaghan and Randall, 1976). Identification of the isolate can be performed via various serological techniques like; VNT (Jones *et al*., 1948; Doll *et al*., 1959; McCollum *et al*., 1962., Burrows, 1968 and Shimizu *et al*., 1989), Immunoperoxidase staining (IP)(Whitwell *et al*., 1992), Radial Immuno diffusion enzyme assay (RIDEA) (Gradil and Joo, 1988), direct and indirect IFAT (Gunn, 1992), also type specific n-PCR (Borchers and Slater, 1993) or nested multiplex PCR (Wang *et al*., 2007) can be used to confirm viral identity.

MATERIALS AND METHODS

Animals and samples

A full molecular identified two EHV-4 PCR positive samples with a Genbank accession numbers of: KP699582, KX866264 were used in the current study. The samples were obtained during an outbreak of abortion in a stud for Arabian horses in Egypt during foaling season of 2014-2015. The Samples of a fetal horse liver were treated separately and aseptically, allocated in sterile specimen containers, chilled, transferred immediately to the laboratory and stored in pieces in liquid nitrogen, then were grounded in a mortar containing suitable amounts of sterile sand and PBS till complete homogenization. Tissue homogenate was subjected to 3 cycles of freezing and thawing before being centrifuged at 3000 rpm for 15 minutes. Supernatant fluid was then collected in a clean sterile tubes where 5x of antibiotic-antimycotic solution were added and incubated for 1 hour. The tubes were immediately frozen at -85°C till use.

Isolation on Cell culture

An aliquot from the previously prepared two tissue homogenate samples (PCR positive for EHV-4 and full identified) were used for viral isolation on MDBK cell line (NBL-1) (Median Derby bovine kidney) (ATCC-CCL-22) with nutrient media of MEM\ Earl's salt (SIGMA), Newborn Calf Serum (BIOWEST) and Penicillin- Streptomycin antibiotic solution (Caisson). Isolation protocol procedures were done according to OIE guidelines (OIE Terrestrial Manual 2013); a recently prepared cell monolayers in 25 cm2 tissue culture flasks are inoculated with 0.5 ml of the Prepared Samples, then was gently rotated to disperse the inoculums over the cells monolayer. The virus is allowed to attach by incubating the inoculated monolayers at 37°C on a platform rocker for 1.5–2 hours. Monolayers of un-inoculated control cells should be incubated in parallel with sterile transport medium only, then, inoculate was removed and the cells were rinsed twice with phosphate buffered saline (PBS). After addition of 5 ml of supplemented maintenance medium (MEM containing 2% serum and twice the standard concentrations of antibiotics, the flasks are incubated at 37°C and inspected daily by

microscopy for the appearance of characteristic EHV CPE (Cell aggregation, focal rounding, increase in refractivity, and detachment of cells)**.** Inoculated flasks then are subjected to 3 cycles of Freezing and Thawing for Harvesting of the Propagated Virion; Contents are thoroughly mixed, placed in a 15ml sterile conical tube and Centrifuged at cooling Centrifuge at 1500 rpm\5 min, Supernatant was collected, stored in small aliquots at -80 to avoid decreasing of virus titer**.** Another Successive 5 serial passage starting by first passage harvest were done with the same procedures, final passage harvest was stored also in small aliquots at -80 for further Identification processes.

Identification of EHV-4 isolate by indirect IFAT

It was carried out using MDBK cells to identify specific fluorescence of EHV-4 using specific and potent polyclonal antiserum to EHV, prepared in horse, provided to Virology research laboratory, Animal Health Research Institute for this purpose by the National Veterinary Services Laboratories of the United States Department of Agriculture (USDA). The antiserum cross-reacts with EHV-4 and hence is not useful for serotyping. A secondary anti horse IgG antibodies conjugated with FITC was used (Sigma Aldrich).

MDBK cell line was grown on Lab-Tek Glass Chamber Slides, then were inoculated with 50µl\well of suspected isolate from the 6th passages and incubated at 37 C0 for 1 hour for virus adsorption, then 200µl\well of maintenance media was added to each tube and incubated at 37 C0 overnight. The maintenance media was discarded; Slides were rinsed three times with PBS and fixed in cold acetone 100% for 30 min., then acetone was discarded, Slides were air dried. (MDBK intact uninfected cells were treated as above as negative control).

EHV positive Control Antisera was added by 30µl\well, incubated for 20-30min. at 37 C0, excess of Antisera was discarded, Slides were rinsed 3 times with PBS, air dried, and then conjugate was diluted 1\300 and added by 30µl\well also, incubated for 20-30min. at 37 C0, Excess was discarded, Slides were rinsed 3 times with PBS, air dried.

Evans blue counter stain was freshly prepared at 1% concentration, and added with 30µl\well volume, incubated for 20min. at 37 C0, Excess of the dye was discarded, Slides were rinsed 3 times with PBS, air dried.

Slides were examined for EHV Fluorescence under Olympus Fluorescent Microscope with 20 X fluorite lens. (Gunn, 1992)

Identification of EHV-4 isolate by AGID

EHV-4 isolate was tested for identification by Immuno precipitation in 1% Nobel agar using the previously mentioned reference control EHV antisera.

50µl of Suspected EHV Isolate was put in Central well, the same vol. of EHV positive control Antisera was put in 3 Peripheral wells, and other rosette wells were filled with PBS as negative Control. Plates were covered, incubated at 25 C0 at humid atmosphere to avoid dryness, inspected daily\3-5 days for the presence of specific Immuno precipitin. (Crabb *et al*., 1995)

Identification of EHV-4 isolate by nPCR

Viral DNA extraction

Wizard® SV Genomic DNA Purification System (Promega, USA) kit was used for EHV DNA purification following the manufacturer instructions with minor modification of an overnight incubation of each 20 mg-liquid nitrogen frozen liver sample with 275 µL digestion mix at 55oC. Control viral DNA extractions followed the manufacturer's cell culture protocol without the modification. The final

clear lysate was processed according to the manufacturer instructions and stored each in about 70 µL aliquot at -86oC till used.

Identification of EHV-4 by nested-PCR

The OIE reference nested-polymerase chain reaction (n-PCR) EHV-4 580 bp specific glycoprotein B (gB) primers synthetized in METABION, GmbH, USA were used for amplifying the extracted PCR products. Following Emerald Amp, Takara-Bio, Japan instructions, each 5 µL viral DNA extract was initially amplified by the primer sets BS-4-P1 (5'-TCT ATT GAG TTT GCT ATG CT-3', nucleotide positions 1705-1724) and BS-4-P2 (5'-TCC TGG TTG TTA TTG GGT AT-3', nucleotide positions 2656-2637). Generation of the PCR products was performed by GenAmp PCR System 9700 thermocycler following one hold step at 94oC for 10 min. for primary denaturation; 40 cycles of 94oC for 60 sec for denaturation, 60oC for 60 sec for primer annealing and 72oC for 3 min. for extension; one hold at 72oC for 10 min. for final extension and 4oC for cooling. Using 10% 10 µL first PCR product each, the primer sets BS-4-P3 (5'-TGT TTC CGC CAC TCT TGA CG-3', nucleotide positions 1857-1876) and BS-4-P4 (5'-ACT GCC TCT CCC ACC TTA CC-3', nucleotide positions 2456-2437) were used for the secondary amplification run following the initial thermo-profile amplification program. Agarose gel genomic DNA product identification succeeded the standard procedures.

Titration of EHV-4 identified isolate

Titration of EHV-4 identified isolate was performed using MDBK cell line According to Pierre and Michel (1993). Serial tenfold dilutions of isolate were prepared in sterile maintenance medium supplied with 2% Newborn Calf serum, beginning from dilution 10-1 to 10-8 (Dilution\Column) in separate dilution plates. Serial diluted Isolate were transferred by Multichannel Micropipette by 100µl\well on Freshly Prepared MDBK Confluent monolayer in sterile 96 Well T.C plate. Positive Control Non-diluted Isolate was Included in the test, also negative control non-infected Cells was included at 2 last columns. The plates were incubated at 37oC for virus adsorption, and then 200 µl of maintenance media were added to all wells and incubated at 37oC for 3-5 days. Plates were examined daily microscopically for the presence of CPE for each individual well for 3-5 days. The end point was determined and the virus titer was calculated according to the method of Reed and Muench (1938).

RESULTS

Isolation of EHV-4 on cell culture

- Six successive serial passages were carried out on MDBK cell line, Inoculated flasks were inspected daily for specific EHV-4 CPE which developed after 6 days for the first passage, ran short till developed after 2 days for last passage as described in pictures below.
- The obvious CPE which developed on cells started with focal rounding within 48h then aggregation of foci forming a grape like aggregation and giant cell formation ending with cell detachment and necrosis, while Herpes group specific I\N inclusions (Cowdry Inclusion Bodies) were not observed in inoculated cells. The following microscopic images demonstrates the progression of EHV-4 CPE on MDBK cell line.

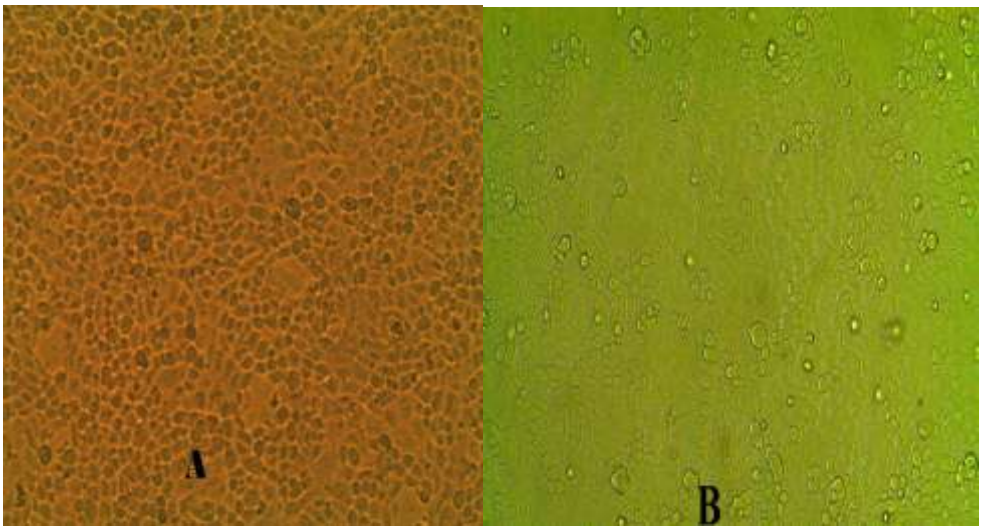

Fig. 1: Microscopic images taken by LEICA® microscope camera at 100x magnification power to MDBK cell line before and after Inoculation of EHV-4 Molecular identified samples, image (A) represents a pre-inoculation stage or control cells 0 day; image (B) demonstrates an increase of cell aggregation with grape like formation as pointed to with black arrow which a characteristic CPE to EHV

Identification of EHV-4 Isolate by IFAT

- IFAT Was carried out 24h infected MDBK cell line for detection of specific fluorescence of EHV-4 suspected Isolate after complete six serial passages.
- Slides showed specific fluorescence indicating presence of the virus as clarified in images.

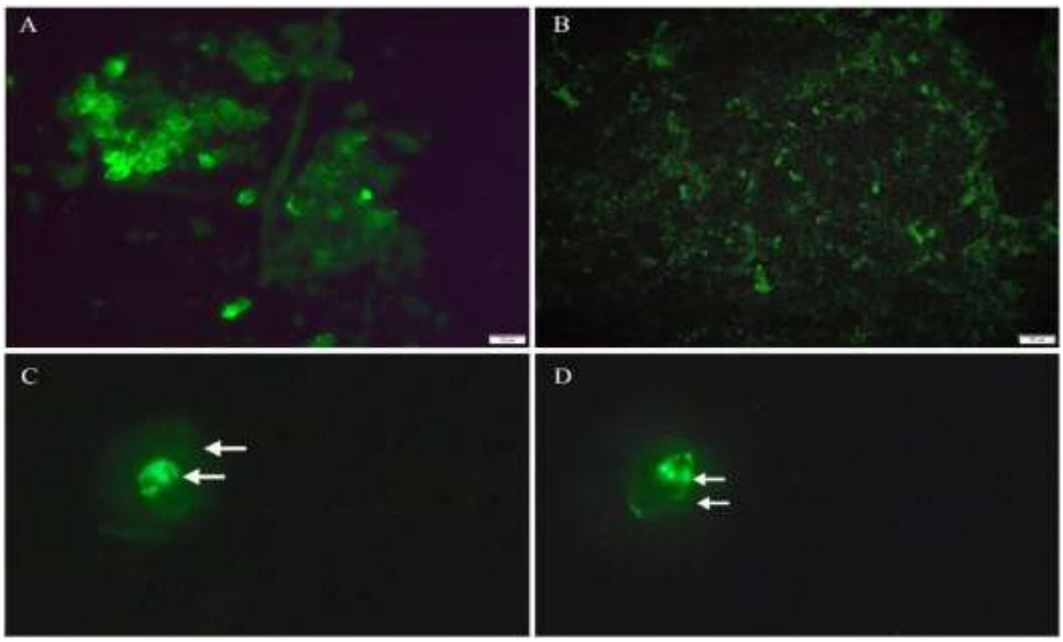

Fig. 2: Different microscopic images taken by Olympus fluorescent microscope camera with 230nm filter to Labtek glass chamber slides contains 24hr infected MBBK cell line with suspected isolate of EHV-4, images are showing specific fluorescence in the infected cells indicating presence of the virus, image (A) was taken at 100x magnification power, while image (B) was taken at 400x magnification power.

Identification of EHV-4 Isolate by AGID

AGID test was carried out as a second step for identification of EHV-4 suspected Isolate. The test showed clear specific precipitation lines as illuminated in figure 3.

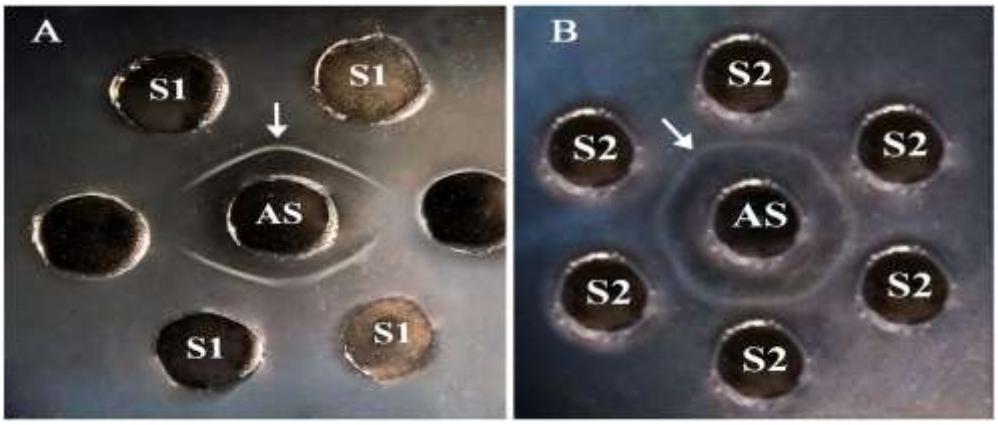

Fig. 3: Pictures for AGID test showing clear distinct precipitation lines between wells Pointed to with orange arrow, image (A) represents the first isolate, image (B) represents the second one; both images contains suspected isolate in the peripheral well as shown on the plate layout and the central wells containing positive control antisera, Specific precipitation lines indicates positive result for Identification of suspected EHV-4 Isolates

Identification of EHV-4 Isolate by Nested PCR

nPCR was carried out as a final test for Identification of suspected EHV-4 Isolate which showed specific M.W bands after analysis by electrophoresis as described in figure 4 which Confirm Identity of EHV-4 Isolate

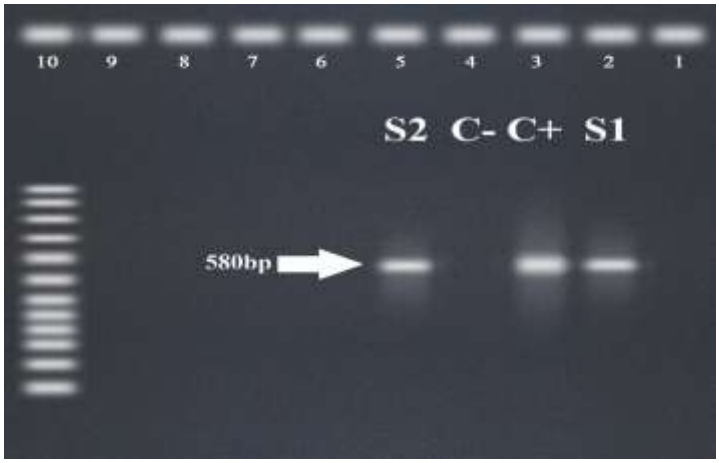

Fig. 4: Image of Agar gel Electrophoretic analysis result of nested PCR of glycoprotein B gene in suspected isolates of EHV-4, showing positive amplification of specific 580bp fragment the two samples in lanes 2, 5 respectively with specific M.W band at 580bp as pointed to with the back arrow as compared with positive control reference virus in lane 3 which confirm identity of EHV-4 Isolate.

Titration of EHV-4 Isolate

Titration of EHV-4 identified Isolate was carried out on MDBK C.C. after complete 6 serial passages, after calculation by Reed and Muench method, EHV-4 Isolate titer was $10^{6.75}$ after completing 6 serial passages.

DISCUSSION

The aim of the research was to use a full molecular characterized EHV-4 positive samples in preparation of a full identified seed viral isolate of EHV-4 mainly to update the national EHV vaccine produced in Egypt, which contains EHV-1 only, and secondary; to be used later in diagnostic purposes.

EHV-4 is not commonly associated with leukocyte-associated Viraemia, (Azab, *et al*.,2012), The reduced pathogenic potential of EHV-4 has been attributed to differences in cellular tropism between EHV-1 and EHV-4, since EHV-4 can only infect respiratory epithelial cells and not vascular EC in vivo (Osterrieder, *et al*., 2010), Under experimental conditions, EHV-1, can infect a broad range of host cells but EHV-4 was confined to primary cell cultures or cells of equine species specially that of respiratory origin (Trapp *et al.*, 2005 and Whalley *et al.*, 2007); However, EHV-4 was successfully isolated on RK-13 (Taouji and coworkers, 2002) and Equine dermal cells (CCL-57) (Hedges, *et al*.,2001 and Diallo, *et al*., 2006).

So we had a challenge in the beginning; that all authors dealt with EHV-4 isolation, differed in cells on which EHV-4 can be cultivated; either primary equine cells only, or cells of equine origin generally, or equine cells of respiratory origin and who split out the rule, succeeded to isolate on RK-13, the challenge was to succeed in isolation of the virus on other cells other than mentioned ones, due to considerable reasons:

1-Unavailability of primary equine cell culture due to lack of source and facilities.
2- Unavailability of cell lines of equine origin in Egypt and very high pricing if imported.
3- Instability of RK-13 cells in VACERA (the only cell lines source in Egypt), and difficulty of handling in lab, beside un-preference of use for EHV-4 due to unclear CPE (OIE Terrestrial manual, 2008).

We made trials on more than a type of cell lines, BHK-21, Vero, RK-13 and MDBK, the later was chosen to complete trials of viral isolation, due to:

1-High stability and easy of cultivation in lab.
2-Slower growth rate other than other types, which matches the nature of EHV of slow growth.
3-More readiness for viral growth and appearance of clear CPE.

CPE was observed, as foci of rounded dark cells, the cell sheet degenerated to small grape like clusters and occasionally small syncytia are formed, these changes become generalized and they destroy the cell sheet finally as in consistence with Bagust and Pascoe, 1968, as observed in Figure: 1

CPE was started in first passage at the 6th day, in contrast to (Bagust and Pascoe, 1968) which recorded CPE after 2-5 days nor at the 4th day as recorded with (McCollum *et al*., 1962a and Kawakami *et al.*, 1962). Which may be due to:

1-Previous recorded incubation periods (till appearance of clear CPE) were carried out on EHV-1, which differs in pathogenic potential than EHV-4 as stated before.
2-Low virus titer in samples used for isolation.

It was observed that incubation period decreased in subsequent passages to reach less than 48 hours in the sixth passage. The virus was adapted and Monolayer tissue cultures have become the major means of propagation, as stated with (Koch, 1967), also with subsequent passages CPE became clearer, like grape like aggregation of cells which is recorded in our results, did not clarified before the 4th passage, and what appeared before that was only focal rounding of cells then unshaped aggregation ended by necrosis and detachment, which is mainly explained due to low virus titer in first few passages. (Weiblen *et al*., 1994).

For confirmation of successful isolation process, viral identification was a must, we carried out a nested PCR as done with raw samples, positive results was recorded which confirm viral identity.

Also, serological testing were done using reference control antisera for EHV as described before, all types of available polyclonal reference antisera are cross reactive between EHV1, 4 due to highly antigenic relationship; thus it can used to confirm identity but can't differentiate between them (OIE terrestrial manual, 2008), which is already done using type specific nested PCR (Wagner, *et al*., 1992).

Starting with IFAT, it was stated that direct immunofluorescent testing provides an indispensable method to the veterinary diagnostic laboratory for making a rapid preliminary diagnosis of EHV (Gunn, 1992; OIE terrestrial manual, 2008).

Also Side-by-side comparisons of the immunofluorescent and cell culture isolation techniques on more than 100 cases of equine abortion have provided evidence that the diagnostic reliability of direct immunofluorescent staining of fetal tissues obtained at necropsy approaches that of virus isolation attempts from the same tissues (Gunn, 1992).

That was the next challenge, that previous researchers incurred that test, recommended direct method; which was not available in our lab, instead we used indirect method using reference control horse antisera beside reference anti-horse IgG antibodies conjugated FITC (SIGMA).

Also we developed the test to be carried out in Lab Tek® glass chamber slides instead of conventional cover slips or Leighton tubes.

In fact, results were good and promising with clear positive specific fluorescence with absence of nonspecific reactions due to use of Evans blue counter staining, as observed in Figure: 2.

Moving to AGID, no previous researchers on EHV have mentioned the former test in sero-confirmation of viral identity (Thomson, *et al*., 1976; Allen, *et al.*, 2000 and OIE terrestrial manual, 2008), it was a challenge to try the test in our research and validate its results by another serological tests to be used later in other researches in same issue, positive results was recorded in the 2 tested isolates as described, test was repeated 3 times on the same samples and the origin raw samples showing the same result with clear distinct precipitation lines, also negative standard samples were tested with negative results recorded, as observed in Figure: 3.

VT was carried out then on EHV-4 isolate after nearly finishing identification steps, as described in 4.9, titer of EHV-4 in 6th passage was $10^{6.75}$, which was a satisfying titer reliable for next steps in our research.

CONCLUSION

EHV-4 is a serious pathogen that infect horse populations on all continents, unlike what is known before that it is confined to URT infection; EHV-4 was detected in abortion outbreak in an Egyptian horse stud at 2015, also EHV-4 was identified after isolation on MDBK cells which is considered the first successful isolation trial on such cell type, being a clear alteration of pathogenic potentiality of the virus.

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