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Studies on molecular diagnosis of FMDV during 2006 outbreak in Egypt

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ABSTRACT

At the beginning of 2006, vesicular condition of the feet and buccal mucosa stroked cattle and buffalos in Egypt. The disease was diagnosed clinically as Foot-and-mouth disease (FMD). Serological tests revealed that the epidemic was due to FMD virus (FMDV). Therefore molecular assay was pre-requisite to rapidly and precise identification of the causal agent of the disease. RT-PCR assay was used and confirmed that the causative agent is FMDV serotype 'A'.

INTRODUCTION

Foot-and-mouth disease (FMD) is the most economically significant animal viral disease worldwide. It can cause vesicles in the mouth, on the nose and on the coronary bands of the hoofs. Milk production drops dramatically and animals become lame. Mortality can be high in young animals in unvaccinated herds. Trade of livestock and animal products must be blocked and the export of agricultural products must be banned from areas where the disease occurs *(1)*.

Seven FMDV standard types O, A, and C are prevalent in all continents where the disease occurs, SAT1 is found in Africa and Asia, and SAT2 and SAT3 are limited to Africa, whereas Asia1 occurs only in Asia. These limitations are due to the pattern of the meet trade than to any inherent properties of the serotypes. Overall, outbreaks of types O and A occur more frequently than the other types *(2)*.

FMD is enzootic in Egypt and outbreaks have been reported since 1950. The disease virus serotypes SAT2, A and O were last reported in the years 1950, 1972 and 2000, respectively. Virus type O was incriminated in the last two epidemic outbreaks, which occurred in 1987 and 1993 *(3)*.

In the past few years, RT-PCR has been used for the diagnosis of FMD *(4-7)*. However, its use for serotyping of FMDV is limited and involves much labor *(8-10)*.

With the dawn of 2006, a massive suspected FMD outbreak struck the Egyptian veterinarian, scientists and authorities. The precise and final diagnosis consumed too much time that result in more economic losses. Consequently, diagnostic test procedures should be rapid, sensitive and specific *(11)*. In addition, the identification of the serotype and its antigenic characterization is important in vaccine selection and proper industrial formulation for the proficient containment of the disease.

Therefore, the study approached to molecular identification of the isolates from FMDV suspected clinical samples during the disease outbreak in Egypt 2006.

MATERIALS AND METHODS

1. Epithelial tissue samples

Clinical symptoms of suspected FMD were observed on bulls in the quarantine station of Ismallia Governorate in January 2006. The signs appeared rapidly in other regions of Lower and Upper Egypt especially in the farm of Faculty of Agricultural in Assiut where the symptoms clinically diagnosed as foot-and-mouth disease (Personal communication). Suspected FMD epithelial samples, collected from cattle in Fayoum province and other provinces, were brought to Veterinary Serum and Vaccine Research Institute (VSVRI) in cooperation with Animal Health Research Institute (National laboratory) and General Organization of Veterinary Services (GOVS)), Ministry of Agricultural, Egypt. Foot-and-mouth disease virus was isolated and identified by the conventional methods (virus isolation, C.F.T. and ELISA). Also, suspected epithelial tissues were submitted to OIE/FAO World Reference Laboratory for Foot-and-Mouth Disease (WRL

for FMD), Pirbright, England, for confirmation using RT-PCR and DNA sequencing.

The samples from Fayoum were placed in a transport medium, composed of equal amounts of glycerol and phosphate buffer (glycerol/buffer mixture), and were stored at -70°C until used.

2) Virus isolation in cell line

Firstly, FMDV suspected epithelial samples were inoculated onto tissue cultures. The susceptible used cells were Baby Hamster Kidney cells (BHK 21 clone 13). The cells were supplied by the Animal Virus Institute, Pirbright, UK. They were propagated at FMD Department, Abbassia, Cairo, using Minimum Essential Medium (MEM) with Earl's salts and 8-10% sterile newborn calf serum *(12)*.

3) Detection of the virus in laboratory experimental animal

Unweaned Swiss baby mice were inoculated with samples suspension intraperitoneal *(13)*. Paralysis and death of the inoculated suckling mice indicate positive virus detection.

4) Detection of FMDV by molecular assay:

a) Extraction of nucleic acid

Total RNA purification kit (V-gene Biotechnology Limited, Industrial Zone Xiacheng District Hangzhou China, Hangzhou, Zhejiang 310022, China) was used for extraction of clinical samples as described by the manufacture's instructions.

b) Reverse transcriptase-polymerase chain reaction (RT-PCR)

. Single tube RT-PCR kit (QIAGEN OneStep RT-PCR Kit. QIAGEN, Germany) was used. It was carried out according to the manufacture's protocol to perform the reverse transcription and the subsequent PCR in a single reaction tube. The reaction was done in 50 µl reaction volume, containing 10 µl RNA template, 1 µM from each gene specific primers, 2 µl dNTP (mix), 2 µl enzyme mix, 10 µl 5x buffer and RNase-free water. Thermocycler program was: (1) 30 min at 50°C, (2) 15 min at 95°C, (3) 1min at 94°C, (4) 1 min at 55°C, (5) 1 min at 72°C; repeating steps (3), (4) and (5) for 30 cycles and finally (6) 10 min at 72°C. (-) control specimen containing sterile RNase-free water was included.

In order to achieve molecular detection and typing in one step by RT-PCR of FMDV suspected serotype 'A' during the massive 2006 outbreak in Egypt specific primers (PH1/PH3-PH8) derived from 1D/2B regions *(Table 1)* were used *(14)*. All primers were synthesized by Metabion, Germany. The expected size of amplified products by primers combination was 732 bp.

c) Agarose Gel Electrophoresis of PCR Products

Samples were prepared on parafilm. 2 µl loading buffer + 10 µl PCR product were mixed. Molecular weight marker was also prepared. Load samples into the wells formed in the gel.

. Electrophorese at 100 volts for 20 min (minimum) or 10 volts overnight.

. View and photograph the gel on an UV-transilluminator. Use UV-safety spectacles.

Result and Discussion

The massive foot-and-mouth disease outbreak that traumatized Egypt, veterinarians and veterinary authorities in January 2006 spread rapidly causing severe economic losses in calves, dairy farms and fattening animals with subsequent increase in prices of milk and meat. The primary diagnosis of FMDV samples with traditional methods in our lab and virus serotyping that was supported by WRL for FMD, Pirbright, England depending on conventional methods and most importantly nucleic acid amplification assays and DNA sequencing for precise diagnosis, typing and tracing the origin of disease, consumed too much time. Thus, the necessity to own our tools for rapid, accurate and sensitive detection and identification were a must. Therefore, molecular study of FMDV and choice of nucleic acid amplification oligonucleotides targeting the outbreak isolates were needed.

Suspension of clinical samples from the outbreak were inoculated onto tissue cultures and in unweaned Swiss baby mice. Isolation of FMDV on BHK-21 was satisfactory with pathognomic cytopathic effect *(Fig. 1)*. In addition, inoculated baby mice suffered paralysis and death *(Fig. 2)*.

RT-PCR assay was performed on the epidemic samples to determine the serotype of FMDV using primers set PH1/PH3-PH8 of which sequences had originated from 1D/2B gene regions. The overall results of the assay indicated that the causative agent would be FMDV serotype A *(Fig. 3)*.

Degenerate primer (PH3-PH8), which is a mixture of similar primers that have different basses at the variable positions, specific for serotype A was used to overcome sequences variation and subtypes within serotype A. More than 10 genetic groups of FMD serotype A viruses ***(15, 16)*** and 32 subtypes of serotype A ***(13)*** have been identified. In addition, comparison of almost 300 complete, or nearly complete VP1 sequences has shown that type A viruses can be grouped into three major geographically restricted genotypes, (i) Euro-SA; (ii) Asia and (iii) Africa, although occasional spread between these continents may take place ***(17)***.

Foot-and-mouth disease world reference laboratory (FMD-WRL) reported that FMDV strain A/EGY/1/2006 virus with 1D (VP1) gene length of 639 nt, depending on unrooted neighbor-joining tree based on a comparison of the complete VP1 gene, belonged to Africa topotype and considered to be more genetically related to A/KEN/15/98, A/ETH/7/92, A/ETH/23/94, A/ETH/1/94 and A/YEM/2/98 while diverged from A/EGY/1/72 (Data not shown). Thus, serotype A was probably introduced into Egypt from live trade in FMDV persistent infected cattle with the African horn countries. The suggestion is consistent with previous work ***(2)***, which cited that in countries where general vaccination is practiced every year, outbreaks are usually associated with different strains imported in carrier animals or infected meat.

Acknowledgement to molecular study and bioinformatics of FMDV, the specificity of the primers (PH1/PH3-PH8) for amplification of VP1 gene of serotype A was believed. All the specific primers (PH1/PH3-PH8) sequences were aligned with nucleotide (nt) sequence database at the National Centre of Biotechnology Information (NCBI) site using the Basic Local Alignment Search Tool (BLAST) programs ***(18, 19)*** where the virus/serotype was confirmed (Data not shown).

The specificity of the candidate amplified fragment of 1D gene, used in this study, to FMDV serotype A was also supported by Mohapatra et al. ***(20)***, who identified a region spanning 409-429 nucleotide (located inside the candidate fragment used in this study) in 1D region alignment and found this stretch to be variable among FMDV serotype A genotypes (at least by 10 nucleotides) but conserved within a genotype.

In this study, the nucleotide position, in correspondence with the numbering of the FMDV strain O_1 K ***(21)***, of the forward primers specific to the virus 1D gene of A was 3124 (VP1 nucleotide 128, residue 43), ***(14)***. The positions of the used oligonucleotides considered critical points as would be illustrated latter in the antigenicity, immunogencity and phylogenetic studies of FMDV.

Seven distinct serotypes and >60 subtypes of the virus have been characterized by serological analysis. The major antigenic determinants are located in a single viral protein, VP1 ***(22)*** and potentially immunogenic regions have been identified in the C-terminal third of this protein ***(23)***.

From the homology to the O_1 K nucleotide sequence, it was concluded that in FMDV C_1 and FMDV A_5 the VP1 coding sequences start with nucleotide 2977 and stop at position 3603 and 3612 in C_1 and A_5, respectively. Hence, it follows that the length of the VP1 coding region varies slightly between the different serotypes of FMDV: the O_1 K nucleotide sequence codes for a protein of 213 amino acid residues and the sequences of types C_1 and A_5 code for 209 and 212 amino acids, respectively ***(24)***.

The region of the VP1 gene for A subtypes between codons 130 and 171 has a high degree of variation and also codes for an important immunogenic site on the viral surface. Consequently, nucleotide changes in this region are most likely involved in the appearance of new antigenic variants ***(25)***.

Isolates of different serotypes differ at two highly variable regions, amino acid positions 42-60 and 134-158, but isolates of the same serotype show major differences only

in the variable region between amino acids 134 and 158. Since the remaining amino acid sequence of VP1 is highly conserved, it was concluded that 134-158 amino acid variable region is involved in subtype specificity, whereas both variable regions contribute to serotype differences *(26)*.

In addition, the sequences spanning the 250 nt of the FMDV VP1 3' end (positions 400 to 650), extensively used for the virus phylogenetic analysis, showed a lower informative content. In spite of this, the use of sequences from this region allowed the derivation of phylogenetic trees of type A and type O FMDVs, which showed topologies similar to those previously reported for the whole VP1 gene *(27)*.

Lastly, Molecular amplification of 1D and 3D coding regions confirmed that the 2006 outbreak in Egypt was due to FMDV serotype A (Africa topotype). The outbreak posed the crucial need to develop rapid and reliable laboratory diagnostic procedures to support more efficient disease identification and control. It also demonstrated the slowness and limitation of the traditional diagnostic techniques (virus isolation, complement fixation test and ELISA).

Finally, the proficient application of molecular biology and diagnostic techniques in our country will facilitate accomplishing rapid FMDV identification in the field with effective containment of the disease in susceptible areas using robust vaccine formula besides the evaluation and the periodical confirmation of the serotype strains which formulate the new applied bivalent vaccine in Egypt. In addition, it will protect the poor community from more socio-economic earthquakes accompanied with virus outbreaks like that occurred by foot-and-mouth disease virus and avian influenza virus emergency in 2006 in Egypt.

Table 1. FMDV-specific primer sequences.

Primer	Orientation	Sequence (5' to 3')	Serotype specificity	Genomic location
PH1	Reverse	AGC TTG TAC CAG GGT TTG GC	All serotypes	2B
PH3	Forward	GTC ATT GAC CTC ATG CAG ACC CAC	A	1D
PH4	Forward	GTT ATT GAC CTC ATG CAG ACC CAC	A	1D
PH5	Forward	GTC ATT GAC CTC ATG CAC ACC CAC	A	1D
PH6	Forward	GTC ATT GAC CTC ATG CAG ACT CAC	A	1D
PH7	Forward	GTC ATT GAC CTC ATG CAA ACC CAC	A	1D
PH8	Forward	GTC ATT GAC CTT ATG CAG ACT CAC	A	1D

.The expected sizes of amplified products by primer pair combinations: PH1/PH3-PH8:732 bp *(14)*.

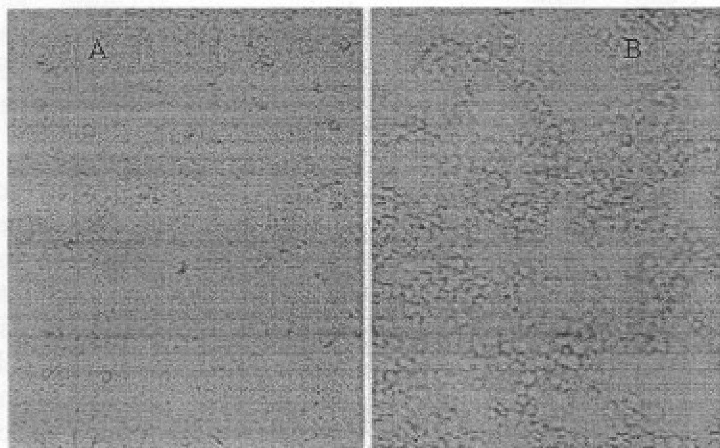

Fig. 1. Cell culture (normal and infected cells) used in FMD virus isolation. A) Normal BHK21 cells; B) Infected BHK-21 cells. The photographs were acquired through 10 objective lens.

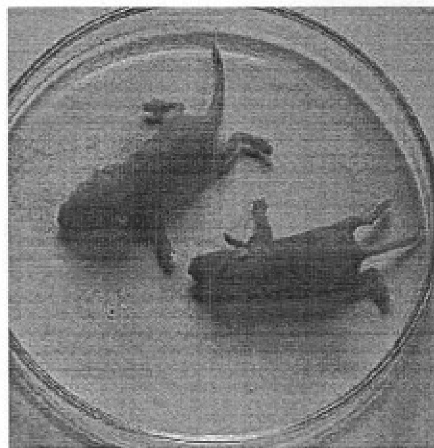

Fig. 2. The unweaned baby mice show paralysis in the limb.

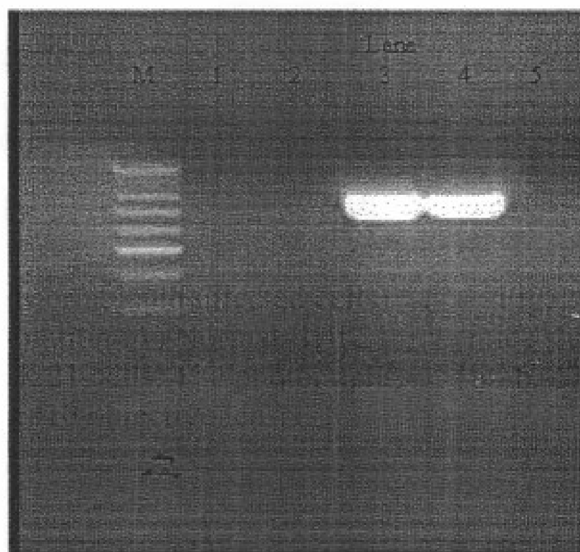

Fig. 3. RT-PCR for identification of FMDV serotype A. Primers mixtures PH1/PH-PH8 were used for targeting 1D region of serotype A RNA templates. M: 100 bp ladder (100, 200, 300, 400, 2*500, 600,......1000 bp); lane 3 and 4: positive FMDV RNA with 732 bp amplicon for FMDV serotype A 1D gene; lanes 1, 2 & 5 negative control samples.

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الملخص العربى

دراسات عن التشخيص الجزيئى لفيروس مرض الحمى القلاعية أثناء وباء ٢٠٠٦ في مصر

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[١]قسم الفيرولوجى كلية الطب البيطري-جامعة الزقازيق

[٢]قسم الحمى القلاعية معهد بحوث الأمصال و اللقاحات البيطرية بالعباسية- القاهرة

مع بداية سنة ٢٠٠٦ تفشت حالات تحوصل في الفم و القدم في الأبقار و الجاموس في مصر و قد تم تشخيصه إكلينيكيا على أنه مرض الحمى القلاعية. و بعد أيام، أكدت الاختبارات السيرولوجية أن الوباء كان سببه فيروس مرض الحمى القلاعية و بالتالي كان الفحص الجزيئى ضروريا للتعرف السريع و الدقيق على مسبب المرض. ولذلك أستخدم فحص النسخ العكسي و تفاعل البلمرة المتسلسل و أكد أن مسبب المرض هو فيروس مرض الحمى القلاعية الصنف 'A' .