Transboundary and Emerging Diseases

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RAPID COMMUNICATION

Emergence of Foot-and-Mouth Disease Virus SAT 2 in Egypt During 2012

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Summary

The epidemiology of foot-and-mouth disease (FMD) in North Africa is complicated by the co-circulation of endemic FMD viruses (FMDV), as well as sporadic incursions of exotic viral strains from the Middle East and Sub-Saharan Africa. This report describes the molecular characterization of SAT 2 FMD viruses that have caused widespread field outbreaks of FMD in Egypt during February and March 2012. Phylogenetic analysis showed that viruses from these outbreaks fell into two distinct lineages within the SAT 2 topotype VII, which were distinct from a contemporary SAT 2 lineage of the same toptype from Libya. These were the first FMD outbreaks due to this serotype in Egypt since 1950 and required the development of a tailored real-time reverse-transcription PCR assay that can be used in the laboratory to distinguish FMD viruses of these lineages from other endemic FMD viruses that might be present in North Africa. These data highlight the ease by which FMDV can cross international boundaries and emphasize the importance of deploying systems to continuously monitor the global epidemiology of this disease.

Introduction

Foot-and-mouth disease virus (FMDV; family *Picornaviridae*, genus *Aphthovirus*) causes a highly contagious vesicular disease that affects cloven-hoofed animals. FMDV exists as seven antigenically and genetically distinct serotypes [O, A, C, Asia 1, Southern African Territories (SAT) 1, SAT 2 and SAT 3] that can be subdivided into a number of temporally and spatially distributed topotypes. Foot-and-mouth disease is endemic in many countries in Africa, Asia and parts of South America and may cause sporadic outbreaks in FMD-free countries and regions. Foot-and-mouth disease virus serotypes O, A and Asia 1 are endemic or cause periodic FMD outbreaks in the Middle East and serotypes O and A cause FMD outbreaks in North Africa (Knowles et al., 2009; Jamal et al., 2011; Stram et al., 2011). This region is also threatened by sporadic incursions of different topotypes and other FMD serotypes that are normally restricted to Sub-Saharan Africa (Knowles et al., 2007). Between 1964 and 2005, only serotype O was reported in Egypt, with the exception of 1972 when type A was introduced from Sub-Saharan Africa (Knowles et al., 2007). Similarly, widespread outbreaks due to serotype A occurred by importation of infected cattle in 2006 (Knowles et al., 2007).

During 2012, there has been a dramatic upsurge in FMD SAT 2 outbreaks in Egypt. Initial cases were recognized in the Delta Governorates (Gharbia and Sharkia) and Alexandria, and further outbreaks of disease were also suspected in Upper Egypt including Sohag, Qena and Aswan Governorates (Fig. 1). Cattle, water buffalo and small ruminants were affected with severe clinical signs of FMD particularly in young animals where a mortality rate of up to 50% was observed due to multifocal myocarditis. Further to a request by the Egyptian Government, a Food and Agriculture Organization of the United Nations

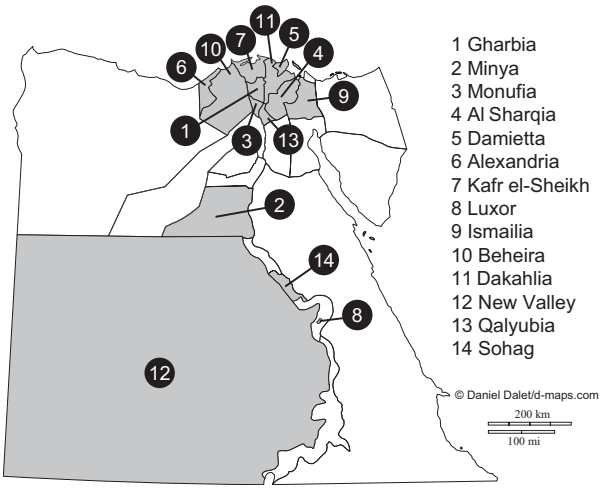

Fig. 1. Locations of foot-and-mouth disease (FMD) outbreaks that have been reported in Egypt. Egyptian Governorates (1–14) are numbered sequentially in order that the FMD outbreaks were reported.

(FAO) emergency team was established to characterize the FMDV strain responsible and assess the field situation with local veterinary authorities with the aim to set up primary containment measures through a national FMD control strategy.

This report describes the use of sequence data to characterize the FMDV recovered from these recent outbreaks in Egypt. Nucleotide sequence data are widely used for phylogenetic analyses to track transboundary movements of FMDV and can also be used to define antigenic determinants of the virus. Furthermore, sequence data were also used to design a sensitive and specific molecular test to detect FMDV from affected animals.

Materials and Methods

This work was undertaken at the Egyptian FMD National Reference Laboratory [Animal Health Research Institute (AHRI)] supported by the FAO World Reference Laboratory for FMD (WRLFMD, Institute for Animal Health, Pirbright, UK). Representative vesicular epithelial samples were submitted to the AHRI from 14 suspected FMD outbreaks (Table 1).

Initial screening of tissue suspensions (Ferris and Dawson, 1988) prepared from these samples was undertaken using an antigen-detection sandwich ELISA kit (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna [IZSLER], Brescia, Italy; Ferris et al., 2011) that detects FMDV serotypes O, A, C and Asia 1, and the SAT 2 component of an antigen-detection sandwich ELISA kit (BDSL, IAH, Pirbright, UK; Ferris and Dawson, 1988). Virus isolation (VI) was also attempted at AHRI using BHK-21 cells, and cell culture supernatants were tested by antigen-detection ELISA (described above). The epithelial suspension samples were also tested by real-time RT-PCR. Briefly, RNA was extracted using QIAamp® viral RNA mini Kit (Qiagen), after which one-step RT-PCR amplification was performed using a Stratagene Mx3005p cycler (Agilent Technologies, Stockport, UK) using conditions previously described (Shaw et al., 2007), but with the primers targeting the 3D region of the FMDV genome (Callahan et al., 2002). Additional conventional RT-PCR (agarose-gel electrophoresis) analyses were undertaken using pan-serotypic primers (1F/1R: Reid et al., 2000) and type-specific primers (see below) that target VP1. The initial genotyping was undertaken at AHRI for SAT 2 serotype using RT-PCR with specific oligonucleotides primers SAT-1D209F [5′-CCA CAT ACT ACT TTT GTG ACC TGG A-3′] and SAT-2B208R [5′-ACA GCG GCC ATG CAC GAC AG-3′] targeting the VP1/2A/2B region. Positive RT-PCR products were sequenced (BigDye Terminator v3.1 Cycle Sequencing Kit on a 3130 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA), and comparative analysis of these partial VP1 sequences for the Egyptian FMDV was carried out using available sequences using Basic Local Alignment Sequence Tool (BLAST) of the National Center for Biotechnology Information (NCBI) database. The sequences were sent to WRLFMD for confirmation.

Epithelial samples collected from 10 Egyptian Governorates were submitted to the WRLFMD (Table 1) for confirmatory diagnostic and phylogenetic analyses. Additional samples from contemporary outbreaks in Libya, Palestinian Autonomous Territories (PAT), Bahrain, as well as older material collected from Kenya (2009) and Cameroon (2005) were also included for comparative purposes. Samples were tested by VI [using primary bovine thyroid cells and the IB-RS-2 cell line (De Castro, 1964)], antigen-detection ELISA (Ferris and Dawson, 1988) and by two pan-serotypic one-step real-time RT-PCR assays that amplify the 3D and 5′ untranslated regions of the FMDV genome (King et al., 2006; Shaw et al., 2007).

Virus isolates were submitted for VP1 sequence analysis using a one-step RT-PCR method as previously described (Habiela et al., 2010). After RNA extraction (RNeasy® kit; Qiagen Ltd., Crawley, West Sussex, UK), oligonucleotide primers used for PCR amplification were either SAT2-1C445F [5′-TGG GAC ACM GGI YTG AAC TC-3′] or SAT2-P1-1223F [5′-TGA ACT ACC ACT TCA TGT ACA CAG-3′] as a forward primer and SAT-2B208R [5′-ACA GCG GCC ATG CAC GAC AG-3′] as a reverse primer. RT-PCR products were sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) using the following oligonucleotides: SAT2-1C445F, NK72 [5′-GAA GGG CCC AGG GTT GGA CTC-3′], SAT2-D [5′-GGT GCG CCG TTG GGT TGC CA-3′],

Table 1. Representative data for samples collected from reported outbreaks analysed in this study

WRLFMD ref. no.	Type	Outbreak place (Governorate)	Date sampled	AHRI lab code	AHRI IZLER ELISA	AHRI SAT 2 Ag det BDSL	Pirbright Ag-ELISA	AHRI rRT-PCR	Pirbright rRT-PCR
EGY/2/2012	SAT 2	Alexandria	17/02/2012	R/179	neg	SAT 2	SAT 2	pos	pos
EGY/3/2012	SAT 2	Gharbia	18/02/2012	R/179	neg	nd	SAT 2	pos	pos
EGY/4/2012	SAT 2	Minya	27/02/2012	R/193	neg	SAT 2	SAT 2	pos	pos
EGY/5/2012	SAT 2	Minya	27/02/2012	R/193	neg	SAT 2	SAT 2	pos	pos
EGY/6/2012	SAT 2	Kafr el-Sheikh	27/02/2012	R/187	neg	SAT 2	SAT 2	pos	pos
EGY/7/2012	FMDV-GD	Monufia	28/02/2012	R/189	neg	nd	NVD	pos	pos
EGY/8/2012	FMDV-GD	Alexandria	29/02/2012	R/205	neg	NVD	NVD	pos	pos
EGY/9/2012	SAT 2	Suez[a]	29/02/2012	R/203	neg	SAT 2	SAT 2	pos	pos
EGY/10/2012	SAT 2	Giza[a]	29/02/2012	R/198	neg	SAT 2	SAT 2	pos	pos
EGY/11/2012	SAT 2	Monufia	29/02/2012	R/208	neg	SAT 2	SAT 2	pos	pos
EGY/13/2012	SAT 2	Qalyubia	01/03/2012	R/214	neg	SAT 2	SAT 2	pos	pos
EGY/14/2012	SAT 2	Gharbia	03/03/2012	R/221	neg	SAT 2	SAT 2	nd	pos
EGY/15/2012	SAT 2	Faiyum[a]	04/03/2012	R/220	neg	NVD	SAT 2	nd	pos
EGY/12/2012	NVD	Al Sharqia	29/02/2012	R/197	nd	nd	NVD	pos	neg

AHRI, Animal Health Research Institute; FMDV, foot-and-mouth disease virus; nd, not determined; FMDV-GD, FMDV genome detected by RT-PCR; NVD, no virus detected.

[a]Additional Egyptian Governorates not highlighted in Fig. 1.

SAT2-1C513cF [5′- CAC CCA CAC AGA CAC ACC GGC CAT GGC-3′] and SAT2-1D209bF [5′- CCA CCT ACT ACT TTT GTG ACC TTG A-3′]. Sequences (from the ABI 3730 DNA Analyzer) were assembled using SeqMan Pro™ (DNAStar Lasergene 10.0, Madison, WI, USA), and complete VP1 nucleotide sequences were aligned using BioEdit 7.0.5.3 (Hall, 1999) and Clustal W 1.83 (Thompson et al., 1994). These alignments were used to construct distance matrices using the Kimura 2-parameter nucleotide substitution model (Kimura, 1980) as implemented in the program MEGA 5.05 (Tamura et al., 2011). Midpoint-rooted neighbor-joining trees were then constructed using MEGA 5.05. The robustness of the tree topology was assessed with 1000 bootstrap replicates as implemented in the program.

Based on the VP1 sequence data generated for these samples, a specific real-time RT-PCR assay was developed for diagnostic use to differentiate between viruses from this new SAT 2 lineages and other FMDV that might be present in the region. The design of this new assay was based on a nucleotide alignment containing SAT 2 sequences from North Africa (Libya and Egypt) as well as sequences for other FMDV lineages that are endemic (or pose threats for incursion) to the region (A/ASIA/Iran-05, A/AFRICA/G-VII, O/ME-SA/PanAsia, O/ME-SA/PanAsia-2, O/ME-SA/Egy-72). The specific oligonucleotides used were forward primer [5′-TGA AGA GGG CTG AGC TGT ACT G-3′], reverse primer [5′-CTC AAC GTC TCC TGC CAG TTT-3′] and dual-labelled (FAM-TAMRA) TaqMan® probe [5′-ACA GAT TCG ACG CGC CCA TCG-3′]. This RT-PCR amplifies a 140-nucleotide (nt) fragment encompassing 3742–3881 nt of SAT 2/EGY/9/2012 (Valdazo-González et al., 2012; GenBank accession number JX014255). This assay was designed to be performed using the same cycling conditions and reagents that are used in the FMDV pan-serotypic diagnostic assays (Shaw et al., 2007). Briefly, 25 μl RT-PCRs contained 20 pmol of each primer, 7.5 pmol of TaqMan® probe, 12.5 μl of commercial mastermix (SuperScript III Platinum One-step qRT-PCR system; Life Technologies, Paisley, UK) and 5 μl of RNA extracted from a clinical sample. One-step reverse-transcription and PCR amplification (Mx3005p; Stratagene) was performed using the following cycling programme: 60°C for 30 min and 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 60 s. The performance of this assay was assessed using RNA prepared from FMDV-positive clinical samples that have been previously characterized using the serotype Ag-ELISA (Ferris and Dawson, 1988) and VP1 sequencing. As well as samples from these recent outbreaks, the validation panel also included representative FMDV from other lineages that might be present in the region.

Results and Discussion

Testing of representative samples collected from FMD cases from Egypt using the ISZLER FMD Antigen ELISA kit generated unexpected negative results. As this assay detects only Eurasian FMDV serotypes (O, A, C and Asia 1), these results provided initial evidence that an FMDV from a SAT serotype was responsible for causing these outbreaks. Subsequent testing of vesicular suspensions (and cell culture supernatants generated from VI) using an established SAT

2-specific ELISA (Ferris and Dawson, 1988) yielded positive results. These were the first reported FMD outbreaks due to this serotype in Egypt country since 1950 and represent a northerly expansion of a serotype that is normally restricted to Sub-Saharan Africa. However, transboundary spread of this serotype is not without precedent: previously, FMD outbreaks in domesticated livestock due to SAT 2 have been reported in North Yemen (1990), Saudi Arabia (2000) and Libya (2003).

Virus diversity is high among SAT serotypes, especially for the SAT 2 serotype that is composed of at least 14 geographically restricted topotypes (Bastos et al., 2003). Phylogenetic analysis (Fig. 2) of VP1 nucleotide sequences demonstrated that SAT 2 FMDVs from Egypt belonged to topotype VII of FMDV serotype SAT 2. Foot-and-mouth disease viruses characterized as belonging to this topotype have been previously sampled in a number of countries in Africa including Eritrea, Uganda, Cameroon, Nigeria, Sudan and Libya. Within this topotype, the Egyptian viruses appeared to comprise two lineages (designated as SAT 2/VII/Ghb-12, which was found in the Egyptian Governorates of Faiyum, Gharbia, Giza, Kafr el-Sheikh, Minya, Monufia, Qalyubia, Suiz, and more recently in PAT; and SAT 2/VII/Alx-12, which was detected in Alexandria Governorate). Across VP1, these two lineages differ by approximately 10% (64–66 substitutions) at the nt level indicating that although they share a close evolutionary history, they are not directly related to each other. These nucleotide differences suggest that either two independent introductions or a single introduction of two lineages into Egypt have occurred. The Egyptian FMDV sequences share the same topotype to FMDV collected during February 2012 in Benghazi, Libya (lineage denoted as SAT 2/VII/Lib-12). However, the phylogenetic analyses (Fig. 2) shows that these viruses are from different genetic lineages that are also distinct from previous FMDV sequences recovered during 2003 in Libya. These findings suggest that separate incursions of SAT 2 FMDV were responsible for these field outbreaks in Egypt and Libya. The immediate source of the FMDV causing these outbreaks is presumably from one or more Sub-Saharan African countries; however, in the light of the sparse sampling of closely related viruses, it is not possible to use sequence data to categorically define the exact origin of these viruses. Further coordination and sharing of sequence data is now urgently required to formally identify transboundary transmission links between affected countries in the region. Complete FMDV genome sequence analyses from these field cases and additional material may provide a suitable approach to reconstruct transmission trees to connect clusters of outbreaks (Cottam et al., 2008). To generate the tools that might be used for high-resolution molecular epidemiology, complete genome sequences of one of the Egyptian SAT 2/VII/Ghb-12 viruses and the closely related virus from the Gaza Strip have been recently determined (Valdazo-González et al., 2012).

Additional FMDV SAT 2 outbreaks have also occurred during 2012 in Bahrain. Surprisingly, phylogenetic analysis showed that the VP1 sequence of the Bahrain belonged to SAT 2/topotype IV (Fig. 2) and was most closely related to viruses occurring in Kenya in 2009. This topotype normally occurs in Kenya and Tanzania and has also been found in Burundi and northern Malawi. Previously, viruses from this topotype have also been present in cattle in Awassa, Ethiopia, in 1989 (Sahle et al., 2007) and in an abattoir in Sana'a, Yemen Arab Republic, in 1990 (in a goat imported from Sudan).

The SAT 2-specific real-time RT-PCR was evaluated for use as a diagnostic tool. This assay was able to detect SAT 2 FMDV from all of the recent outbreaks in Egypt, Libya, and PAT (as well as older SAT 2/VII samples from Eritrea and Cameroon), but did not cross-react with other five FMDV lineages that might be present in livestock in the region (data not shown): serotype O [PanAsia 2 and Egy-72 (vaccine-like)] and serotype A (A/ASIA/Iran-05 or A/AFRICA/G-IV or G-VII). Furthermore, this real-time RT-PCR assay did not detect FMDV (topotype IV) from the recent outbreaks in Bahrain. This assay has been transferred to AHRI to allow SAT 2 (topotype VII) to be rapidly identified and distinguished from other FMDV that might be present in the country.

These data provide useful background information for the control of FMD in the region. Monitoring of incursions of FMDV is particularly important because although vaccination programmes may be in place to control endemic strains (such as in Egypt where O and A serotype vaccine are used), these vaccines will not provide any protection against exotic lineages of different serotypes that might be introduced into a country. In addition to the local impact of these FMD outbreaks upon the livestock industry in Egypt, the spread of SAT 2 into North Africa may pose an increased threat of spread to European nations in the Mediterranean Basin and beyond. As discussed earlier in this report, the spread of a SAT serotype viruses outside of Africa is a situation not without precedent. In addition, previous SAT 1 FMD incursions outside of Africa have occurred in Bahrain from East Africa in 1962. Within a few months, the virus had spread to Iran, Iraq, Israel, Jordan, Lebanon, Syria, Turkey and Greece. The virus persisted in Iran until 1964 and Turkey until 1965. Furthermore, two more restricted incursions of SAT 1 also occurred in 1969–1970 (Kuwait and Saudi Arabia) and 1984 (North Yemen). Clearly close monitoring of the FMD situation in the region is now needed to define risks of future outbreaks, as well as to ensure that control measures are appropriate for this virus strain.

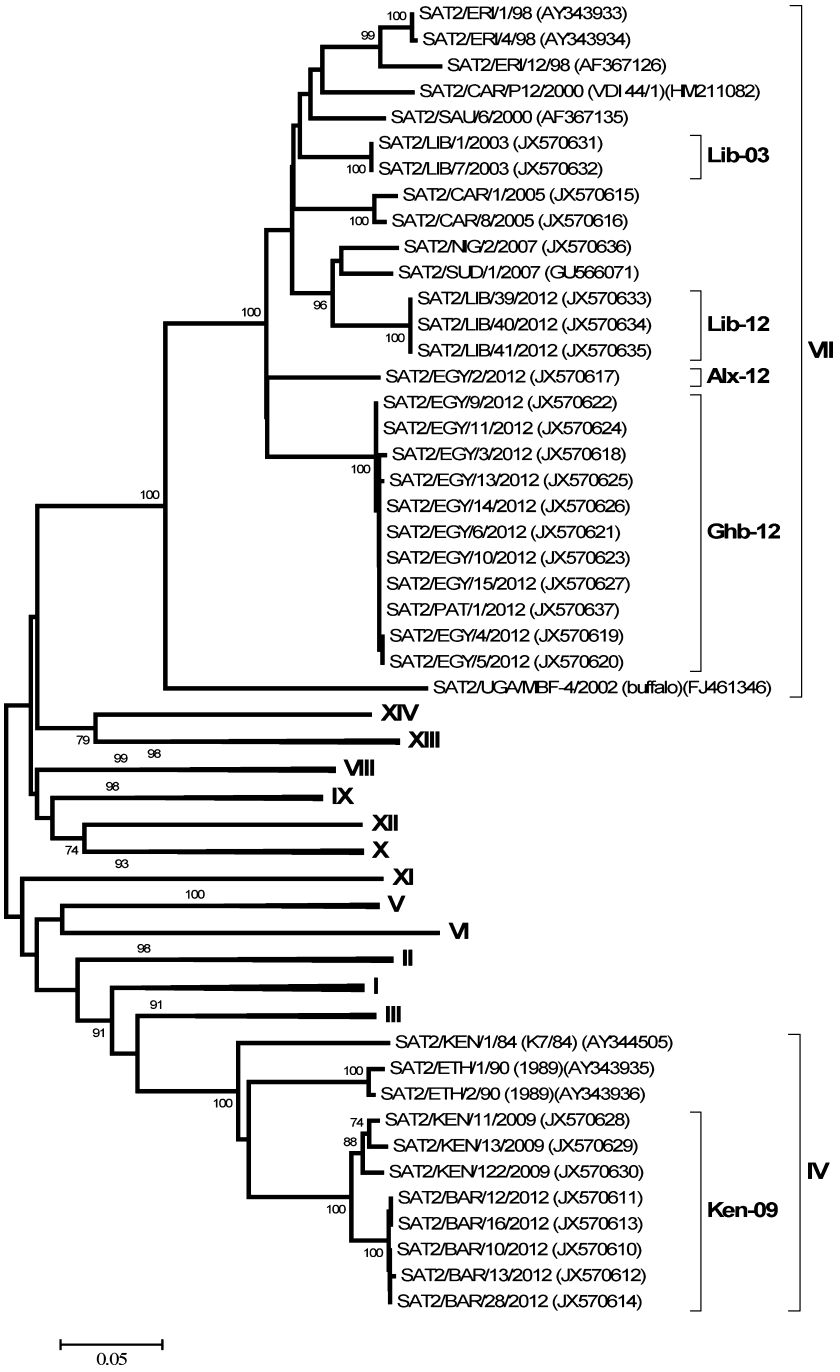

Fig. 2. Midpoint-rooted neighbor-joining tree showing the relationships between the SAT 2 virus isolates from Egypt, Libya, Palestinian Autonomous Territories and Bahrain with other contemporary and reference viruses (GenBank Accession numbers are shown). Roman numerals denote SAT 2 topotypes. Numbers indicate the percentage occurrence of the branches by the bootstrap resampling method.

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