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Research Article

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Identification of Foot and Mouth Disease Virus Strains Originating from Multispecies Susceptible Animals

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Abstract

A first in country trail of detection, isolation and identification of Foot and Mouth disease virus (FMDV) strains was conducted in Qatar during the year 2015-2016. Using rRT-PCR and RT-PCR assay, 31/131 (23%) subject animals were found positive for infection with FMD throughout the investigation period. All of the positive samples were also positive for cultivation demonstrating early cytopathic effect utilizing the uncommonly used Vero cell lines. Genotypic identification trials distinguished five FMDV serotypes namely, A, O, Asia 1, SAT 1 and SAT 3. Five domesticated and wild ungulate animal species were shown infected with these serotypes. Interesting enough, mixed FMDV serotype infections were demonstrated into 4 susceptible animals representing 3 different species. While best to our knowledge it is the first time identifying five different FMDV serotypes in Qatar, further molecular analyses are recommended to confirm the findings, determine the molecular epidemiology of the isolates and to hint the nature of the expectant future vaccine strains.

Keywords

Foot and mouth disease virus; Identification; RT-PCR; Qatar

Abbreviations: FMD: Foot and Mouth Disease; FMDV: Foot and Mouth Disease Virus; rRT-PCR: Real Time Reverse Transcription Polymerase Chain Reaction; RT-PCR: Reverse Transcription Polymerase Chain Reaction

Introduction

Foot-and-mouth disease (FMD) is a highly contagious viral disease affects domestic and wild cloven-hoofed animals including cattle, sheep, goats, pigs, and buffaloes [1,2] leading to devastating economic losses [3]. Over 70 wildlife species were also reported to be susceptible to FMDV [4]. Classified as an *Aphthovirus* belonging to the family *Picornaviridae*, FMDV is genetically constituting seven distinct serotypes namely, O, A, C, Asia 1 and Southern African Territories (SAT) 1, 2, 3 [5-7]. None of which is known to cross-protect each other [8,9].

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Region wise and with specific reference to all seven serotypes, FMD was known to be endemic throughout the Middle East and some Arabian Peninsula countries [7,10,11]. Although Qatar is considered under circulating serotype O [12]; it is also listed within O, A, Asia 1 pool endemic central/east Asia countries [13]. Given that the total estimate of FMD-susceptible animals is 761880, 98% of which are audible. This situation has obligates the demand to import live animals and animal produces to cover the food deficit created by the current 1.8 million estimate of inhabitants with annual increasing rate of 4.7% [14], a condition that would pose risks owing to the increased possibility of introducing and perpetuating of new FMDV serotypes in the country.

Aiming at investigating the current situation of FMD among the susceptible animal species in Qatar, the authors are targeting to establish a first in-country baseline study to routinely detect, conventionally isolate and molecularly identify the existing FMDV field strains, a trial that might offer an opportunity to cast light on the risk poses by the disease and to maintain data about the molecular epidemiology of the circulating field serotypes.

Materials and Methods

Samples and data

A total of 208 samples of whole viremic blood, blood serum, swabs, and tissues collected from 131-FMD-suspected animals representing five species namely; bovine, ovine, caprine, gazelle and Oryx presented at the 8 veterinary centers in Qatar were used in this investigation (Tables 1 and 2). The whole viremic blood samples were immediately chilled and stored at 4°C. Blood serum samples prepared following the standard procedures were stored at -80°C. Fresh tissue samples and swabs collected into 3 mL transport medium each (Puritan® UniTranz-RT™, USA) was stored at -80°C till used. Relevant data were collected and recorded using owner interviewing sample-reporting sheets.

Purification of viral RNA

The High Pure Viral Nucleic Acid Kit, Lot 11858874001, Version 16 (Roche Diagnostic Gmbh, Germany) was used for purification of the viral RNA from each 200 µL whole blood, blood serum and swab elution sample following the manufactures instruction.The QIAamp RNeasy® Mini Kit, Lot 74106 (QIAmp Viral RNA Handbook, 12/2005) was used for purification of viral RNA from each 30g-Buffer RLT-

Table 1: Total number of samples, animal species and percentage of FMD-positive cases in each of the 8-veterinary-centers during the investigation period.

Veterinary Center	Animals	Samples	% FMD-positive/center
Aljamalya	3	7	3%
Alrayan	4	9	3%
Alshahanya	24	27	42%
Alwakara	22	46	7%
Alkhour	23	39	16%
Alrowaise	5	5	0%
Ummsalal	32	57	29%
Alshamal	18	18	0%
Total	131	208	23%

Table 2: Percentage of FMDV-positive samples versus total samples collected from the different animal species presented to the veterinary centers during the investigation period.

Animal species	Total tested animals	Total positive	Percentage
Bovine	38	6	15%
Ovine	71	19	26%
Caprine	12	4	33%
Oryx	7	1	14%
Gazelle	3	1	33%
Total	131	31	23%

digested tissue sample following the manufacturer's instructions. The final 50 µL viral RNA elution prepared from each sample was stored at -80°C till amplification.

Real-time polymerase chain reaction

The qualitative one step real-time polymerase chain reaction (rRT-PCR) assay was applied for primary identification of the FMDV strains using Primer design® genesig® FMDV Kit, Lot J/N 118480-36690 (Genesig Advanced Kit Handbook HB10.01.08, UK). For each 5 µL viral RNA template 2X qRT-PCR one step, 1 µL 1X RT-PCR enzyme mix, and 1 µL 1X FMDV primer probe mix was added to form a final volume of 20 µL. Positive and negative controls were treated the same using 5 µL FMDV reference preparation and 3 µL NTC (Nuclease- Free Water), respectively. Using ABI 7300 Real Time Cycler, amplification followed 42°C for 10 min for primary RT step, 95°C for 2 min for RT inactivation/denaturation, 45 cycles of 95°C for 10 sec and 60°C for 1 min for final amplification.

Reverse transcription polymerase chain reaction

The triple check reverse transcription polymerase chain reaction Lot (K223A) (Genekam Biotechnology AG, Germany) technology was used for detection of FMDV. Each 2 µL RNA template was amplified with 7 µL primer set, 1 µL transcriptase enzyme and 10 µL master mix. Amplification with the thermal cycler followed 42°C for one hour, 70°C for 10 min, and 95°C for 5 min for pre-PCR step; 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec; extra extension step at 72°C for 7 min for final amplification of 131 bp and 197 bp amplicons. All products were subjected to gel electrophoresing analysis and documentation.

Identification of FMDV strains

The Ready to Use PCR Kit: K149-FMDV Genotyping (Genekam Biotechnology, Germany) of 732, 596, 402, 292bp amplicons was used for identification of A, C, O and Asia 1 serotypes, respectively following the manufacture›s guidance. Based on [15,16] Vangrysperre and De Clercq and Callens and De Clercg methodologies, the reverse oligonucleotide P1 FMDV primer sets (Life Technologies) (Table 3) and the forward oligonucleotide Multi-primer mix P126, P130 and P168 of the respective 246, 201, 75bp amplicons was used for identification of SAT 1, SAT 3 and SAT 2 serotypes, respectively. Using approximately 2 µL FMDV positive probe each, all samples were amplified in ABI Thermocycler (Veriti 96-Well Thermal Cycler) following 42°C for 50 min, 48°C for 10min, 70°C for 10 min and 4°C for 5 min for cDNA; 95°C for 10 min, 45 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec followed finally by a cycle of 72°C for 7 min for PCR phase. All products were subjected to gel electrophoresing analysis and documentation.

Virus isolation

Cell culture: The African green monkey (Vero) cell line (American collection) supplied as 70-80% semi-confluent monolayer in 25 cm^3 cell culture flasks (Corning) was used for cultivation of the FMDV strains. Cell lines maintenance was performed by continuous passage in 25 cm^3 cell culture flasks using 4.5 g/L glucose, L-glutamine, sodium pyruvate and $NaHCO_3$ enriched Dulbecco's modified Eagles medium (DMEM) (Lonza, USA) supplemented with 10% bovine calf serum (BCS), 25 µL /mL 1M HEPES (Lonza, USA), 100 IU/mL penicillin, 10 µg/mL streptomycin and 20 µg/mL fungustatin.

Samples: The previously screened positive rRT-PCR FMDV strains were used for isolation using the Vero cell lines. Original swabs collected previously into 3 mL virus transport medium were prepared following spinning for 10 min at 2000 rpm at 20°C. Tissue samples were prepared as 10% homogenates using sterile phosphate-buffered saline (SPBS). All samples underwent filtration using sterile 0.2 µm Millipore filters. Finally, each 2 mL sample aliquot was supplemented with 100 µL 100 iu/mL penicillin, 20 µg/mL streptomycin and 20 µg/mL gentamycin. Then, all aliquots were incubated for 1h at room temperature, preserved at 4°C before inoculation.

Virus cultivation: Each 500 µL pre-prepared FMDV inoculum was inoculated onto 5-day-old 70-80% semi-confluent 25 cm^3 Vero cell line following thrice washing of the cells with 5 mL SPBS. All inoculated cultures were incubated for 1h at 37°C into CO_2-free cell culture grade incubator. Control flasks receiving serum-free DMEM were included and treated the same. Following this, the inocula were removed and each flask received 5 mL 2% BCS supplemented-DMEM. All flasks were incubated back at 37°C and guarded daily for media changing and cytopathic effect (CPE) development.

Results and Discussion

The preliminary rRT-PCR retrieval of 31 FMDV cDNA fragments derived from samples of the 131 subject animals (Tables 1 and 2) indicates susceptibility of these animals to FMD infection, a finding that is in conform with the reported clinical signs of FMD in some positive cases and the results of the confirmatory triple check RT-PCR technique applied in this investigation (Table 4 and Figure 1).

The successful cultivation of the molecularly detected FMDV strains in the uncommonly used Vero cell lines monolayers with production of cell aggregation and vacuolation (Figure 2) and the positive backward rRT-PCR confirmation results would also reinforce the previous detection results. Further, it also sustains the previous findings of Kumar et al. [17] using Vero cell lines to purify viruses. However, the fortunate establishment of infection in this investigation at passage 1 with production of CPE 48 hours post-infection might reflect the sensitivity of these Vero cell lines and/or the replication potentiality of the detected FMDV isolates compared to other strains worldwide. While virus neutralization is standardly used to confirm virus replication in a given cell culture, Strohmaier et al., Moss and Haas and Rodríguez-Calvo et al. [18-20] have already witnessed the sensitivity of the PCR to detect both FMDV and FMDV RNA, findings that could support the use of rRT-PCR as a detecting tool

Table 3: Oligonucleotide primers used for identification of the expected SAT 1, SAT 2 and SAT 3 FMDV serotypes.

Primer	Sequence	Position	FMDV serotype
P1 Reverse	GAAGGGCCCAGGGTTGGACTC	3649 – 3670	All types
P126 Forward	AGGATTGCCAGTGAGACCCACAT	3424 – 3446	SAT 1
P168 Forward	GGCGTTGAAAAACAACT(G/C)TG	3595 – 3614	SAT 2
P130 Forward	TTCGGAAGATTGTTGTGTG	3469 – 3487	SAT 3

Table 4: FMDV field isolate serotypes identified during the investigation period.

Sample No.	Species	rRT-PCR	RT-PCR	Virus isolation	Serotype	Isolate code
1	Bovine	+	+	+	ND	FMDV-BQ15-1
2	Bovine	+	+	+	ND	FMDV-BQ15-2
3	Bovine	+	+	+	ND	FMDV-BQ15-3
4a	Bovine	+	+	ND	A	FMDV-A-BQ15-4
4b		+	+	ND	O	FMDV-O-BQ15-4
5	Bovine	+	+	+	SAT 3	FMDV-SAT3-BQ16-1
6	Bovine	+	+	+	SAT 1	FMDV-SAT1-BQ16-2
7	Ovine	+	+	+	O	FMDV-O-SQ15-1
8	Ovine	+	+	ND	O	FMDV-O-SQ15-2
9a	Ovine	+	+	+	Asia 1	FMDV-Asia1-SQ15-3
9b		+	+	ND	O	FMDV-O-SQ15-3
10	Ovine	+	+	ND	O	FMDV-O-SQ15-4
11	Ovine	+	+	+	A	FMDV-A-SQ16-1
12	Ovine	+	+	ND	O	FMDV-O-SQ16-2
13	Ovine	+	+	ND	O	FMDV-O-SQ16-3
14a	Ovine	+	+	+	O	FMDV-O-SQ16-4
14b		+	+	+	A	FMDV-A-SQ16-4
15	Ovine	+	+	ND	O	FMDV-O-SQ16-5
16	Ovine	+	+	+	ND	FMDV-SQ16-6
17	Ovine	+	+	+	O	FMDV-O-SQ16-7
18	Ovine	+	+	+	O	FMDV-O-SQ16-8
19	Ovine	+	+	ND	O	FMDV-O-SQ16-9
20	Ovine	+	+	ND	O	FMDV-O-SQ16-10
21	Ovine	+	+	+	O	FMDV-O-SQ16-11
22	Ovine	+	+	+	O	FMDV-O-SQ16-12
23	Ovine	+	+	ND	O	FMDV-O-SQ16-13
24	Ovine	+	+	+	A	FMDV-A-SQ16-14
25	Ovine	+	+	+	O	FMDV-O-SQ16-15
26a	Caprine	+	+	ND	O	FMDV-O-CQ15-1
26b		+	+	ND	A	FMDV-A-CQ15-1
27	Caprine	+	+	+	A	FMDV-A-CQ16-1
28	Caprine	+	+	+	ND	FMDV-CQ16-2
29	Caprine	+	+	ND	ND	FMDV-CQ16-3
30	Gazelle	+	+	+	ND	FMDV-GQ16-1
31	Oryx	+	+	ND	ND	FMDV-OQ16-1

* rRT-PCR: Real-time reverse transcription chain reaction.

** RT-PCR: Reverse transcription chain reaction.

*** ND: Not Done

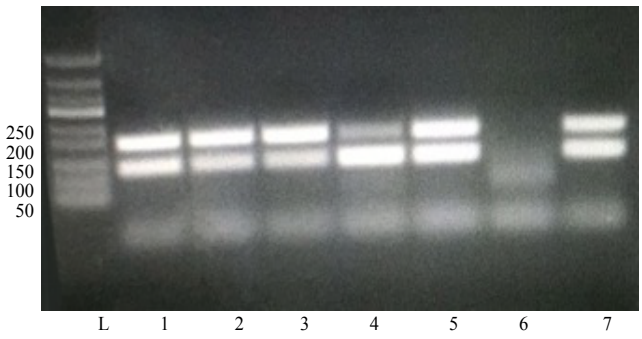

Figure 1: Agarose gel electrophoresis of the amplified gene products of the Triple check RT-PCR aliened to 50bp DNA ladder (L) showing positive FMDV strains at lanes 1-5 corresponding to 131bp and 197bp cDNA fragments with negative and positive controls at lanes 6 and 7, respectively.

in this investigation. Waner [21] has already related the succession isolation of a given virus to the sensitivity of the cell type used. While the replication of FMDV strains in established cell culture systems is well documented [22], cultivation of FMDV in non-established cell cultures was also recently known. Zabal and Fondevila [23] have already demonstrated equal or higher sensitivity of ovine kidney cell culture to FMDV compared to BHK-21 cell lines, the cell lines choice for isolating FMDV. Further replicative studies would expected to give insights about the virus-cell interactions of different FMDV strains using Vero cell lines compared to other culture systems.

The molecular identification trials of the detected and isolated FMDV strains revealed recognition of five FMDV serotypes namely, O, A, Asia 1 (Table 4, Figures 3 and 4), SAT 1 and SAT 3 (Table 4 and Figure 5). The demonstration of non-specific amplicons in lane 2 and 3 of Figure 3 identifying serotypes O and A does not compromises the quality of the test since it is common to display that dealing with some FMDV strains [16]. While the existence of O, A and Asia 1 serotypes were previously reported in the Middle East and Qatar [12,13], to our best knowledge, it is the first time to demonstrate the presence SAT 1 and SAT 3 in the country. With exception to SAT 3, all detected serotypes were previously reported in the Arabian Peninsula [10]

and/or the Middle East [24]. Interesting enough in this investigation, mixed FMDV-serotype infections of Asia 1/O and A/O were detected among 4 animals representing 3 species (bovine 4a, b; ovine 7a, b, 14a, b and caprine 26a, b) (Table 4), a situation that has been reported in previous studies conducted elsewhere in the region [25]. This finding would confirm absence of cross-immunity between the known FMDV serotypes, a fact that was evidenced before [22,26,27] . Without excluding other factors, the finding might also explain the endemic situation of FMD in Qatar from one side and reflect the severity of the clinical features observed among some of the diseased subject animals. Given that out of 28 isolates were subjected to molecular serotyping, 19 (67%) were O, 6 (21%) A and 1 (4%) Asia 1, SAT 1 and SAT 3 each. All of which were of bovine, ovine and caprine origin (Table 4). While molecular tools are recognized to conduct viral characterization [28], further sequence analytic investigations are required to confirm the preliminary serotyping.

Despite the variable percentages of the FMD-positive cases among the involved veterinary centers during the study episode, the detection and isolation of FMDV strains among animals handled by 6/8 (75%) of the total veterinary centers in the country (Table

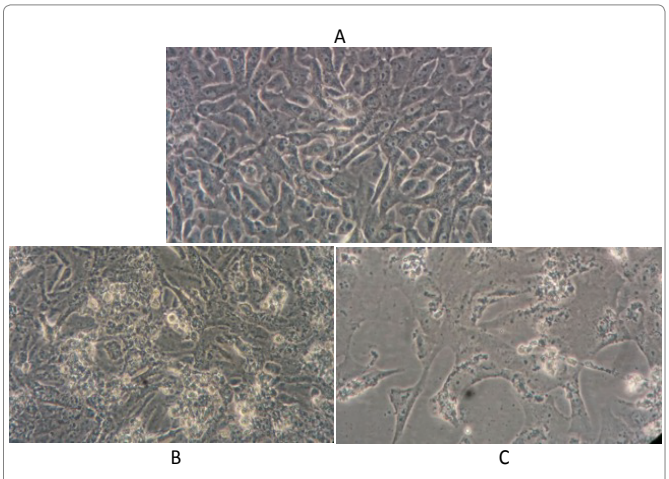

Figure 2: Day-6-postinoculation non-infected-Vero cell line (A) compared to FMDV-O-SQ16-11-infected cells showing cytopathic effect of giant cell formation (B) and cell vacoulation (C) (X20).

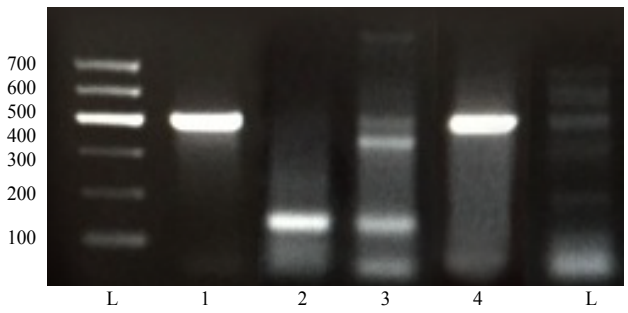

Figure 3: Agarose gel electrophoresis of the amplified FMDV-Q gene products aliened to 100bp DNA ladder (L) showing serotypes O corresponding to 402bp cDNA fragment (lane 1, 3, 4), and serotype A corresponding to 732bp (lane 3). Lane 2 shows non-specific amplicon fragment.

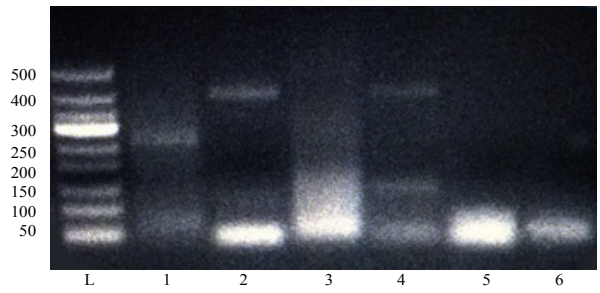

Figure 4: Agarose gel electrophoresis of the amplified FMDV-Q gene products aliened to 50bp DNA ladder (L) showing serotype Asia 1 corresponding to 292bp cDNA (lane 1) and serotype O corresponding to 402bp cDNA fragment (lane 2, 4) isolated from a single sample of an ovine origin.

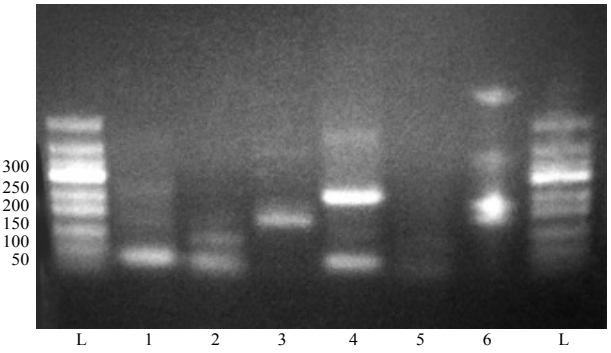

Figure 5: Agarose gel electrophoresis of the amplified FMDV-Q gene product aliened to 50bp DNA ladder (L) showing serotype SAT3 corresponding to 201bp cDNA fragment (lane 3) and serotype SAT 1 corresponding to 246bp cDNA fragment (lane 4) isolated from two different FMD-suspected cases.

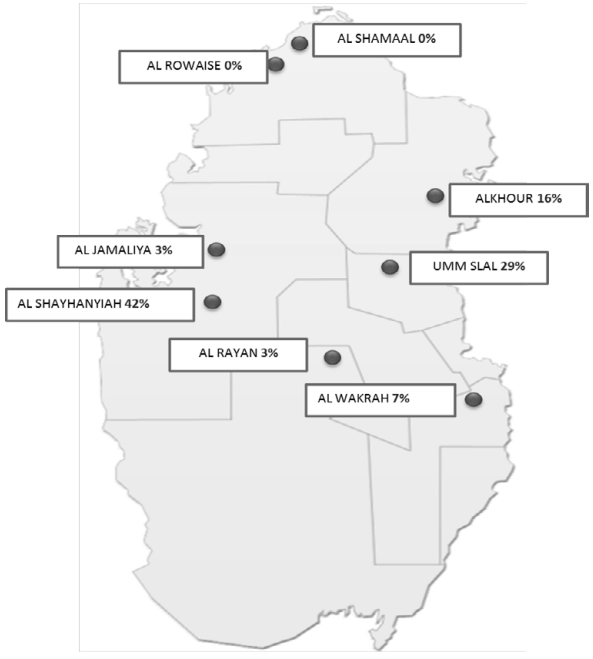

Figure 6: Geographical location of the 8-veterinary centers involved in the investigation (red spots) and the percentage of the total FMD-positive cases in each reported during the study period.

1 and Figure 6) further indicates the endemic situation of FMD in Qatar. Noting that the study based on case reporting rather than representative sampling, failure to detect cases among animals from the two northern veterinary centers, Alrowaise and Alshamal, might reflect the moving wave of the disease during the investigation episode (Figure 6). This observation could be attributed to the geographical location of the animal entry ports south, southeast and southwest the country. The detection of the virus among five animal species namely; bovine, ovine, caprine, gazelle and Oryx (Tables 2 and 4) reflects the wide perpetuation of FMDV strains among the Qatari animal species and the susceptibility of these animals to FMD infection, status that could also reflects the virulence of the disease among some of the domesticated species and the wild ungulates.

The fact that controlling FMD is a worldwide notion rather than an in-country issue alone is well advocated. While this investigation bases for concurrent future efforts to monitor FMD situation through screening of the perpetuating serotypes in Qatar, the deduced results necessitates the veterinary authorities to revise the policy using the current multi-serotypes inactivated vaccine to combat the disease.

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