Establishing a Specific qPCR Assay for Detecting Middle Eastern O Serotype Foot-and-Mouth Disease Virus (FMDV)

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ABSTRACT

The Middle East is one of the main regions under threat of contracting Foot and Mouth Disease (FMD). Indeed, Israel and the Palestinian Territory suffered in the last years from several outbreaks. The FMD viruses responsible for the Middle Eastern outbreaks were predominantly associated with O serotype. Phylogenetic data has indicated that viruses are introduced to the area from different regions, ranging from the Arabian peninsula to the Indian sub-continent. Accurate and rapid identification of the infectious pathogen is essential in endemic areas such as the Middle-East to enable a proper response to combat the disease. In recent years the use of qPCR has become a common practice in the diagnosis of FMDV. A qRT-PCR assay has been developed permitting the discrimination between past and recent Middle Eastern FMDV O type, and the other 6 FMDV serotypes. Moreover, the developed assay, beside, the ability to detect existing strains will probably be able to identify new infecting strains of virus.

Keywords: Foot and Mouth Disease; Middle East; O Serotype; qRT-PCR Assay; VP1 Sequence; Biosecurity

INTRODUCTION

Foot-and-mouth disease virus (FMDV) is one of the most devastating viruses that affect cloven-hoofed animals. The capacity of the virus to spread and to modify its antigenic identity makes it a significant threat to the beef and dairy industries in many countries. One of the FMD-endemic regions of the world is the Middle East, where FMD outbreaks occur almost each year (1). The virus belongs to the genus *Aphthovirus* in the *Picornaviridae* family. The virus genome is an 8.3-kb single stranded RNA in the plus orientation carrying a poly-A tract at its 3' end and a viral genome protein (VPg) at its 5' end (2-4). There are seven different virus serotypes that do not mutually cross-protect, and each comprises of numerous subtypes; about 80 in total (5). The large number of subtypes results from the high rate of mutation, especially in the VP1 gene (6-9). The VP1 gene encodes a structural protein exposed on the surface of the virion which carries the major antigenic sites for the immunological identity of the virus (10-12). The traditional means of protection is vaccination, which greatly reduces the occurrence of the disease (5). Nevertheless, there are hundreds of outbreaks in Asia, Africa, South America and Eastern Europe each year, whereas North America, Australia and Western Europe are virus-free regions and domestic animals there are not vaccinated (13, 14).

The Middle East, including Israel, is one of the main areas where FMDV resides: there are outbreaks almost every year, some of them major with large numbers of infected farms across the country. In recent years the O-type virus has been the prominent type responsible for outbreaks in the Middle East, and throughout the years a large collection of the viruses have been accumulated, enabling the establishment of an Israeli database. The fact that the majority of FMD

outbreaks were of O serotype prompted the establishment of a qPCR protocol utilizing VP1 sequences as templates for the specific detection of O serotype viruses that were responsible for past outbreaks of FMDV in Israel (15).

The aim of this publication was to describe a recently developed assay based on degenerate primers and 2 probes with different fluorophores resulting in a superior probability of identifying a current infectious strain and/or future serotype O viruses entering the Middle East.

MATERIAL AND METHODS

RNA extraction and qPCR

RNA was extracted using Viral Gene-spin (Intron Biotechnology, South Korea) according to the manufacturer's protocol.

Primes and Probes

Primers (Sigma-Aldrich, Israel) and probes (Bioresearch Technologies, USA) sequences are listed in Table 1.

qPCR

A single step RT-qPCR was performed by using qScript XLT mix (Quanta BioSciences, MD, USA) according to the manufacturer's protocol. qPCR systems (primers and probes) were designed and prepared by Biosearch technologies (Biosearch technologies, CA, USA).

Bioinformatics

Multiple homology analysis was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). EMBOSS set of programs were used for other bioinformatics activities.

RESULTS AND DISCUSSION

In order to institute a qPCR able to identify specifically serotype O FMD, full FMDV O viral genome sequences from the Middle East and neighboring countries were used to search for highly conserved sequences suitablefor Taqman assay (Table 1). To assess the ability of the assay to identify FMDV of O serotype, isolates from 1982-2014 were examined. All isolates were successfully detected except for Neve Ur 2010 and Tira 2014 isolates (Table 2).

To reveal whether genomic changes were the cause of the failure to react with Neve Ur and Tira isolates, a ClustalW2 analysis was carried out with Israeli VP1 sequences of recent isolates together with the primers and probe used (Figure 1). This analysis clearly showed an important degree of variability in the probe binding site and to a lesser degree in both primer binding sites resulting in critical changes had to be made particularly in the probe and less so in both which primers sequences. As for the probe, the C at position 5 was changed to T, the A at position 9 was changed to A/G (R), the C at position 14 was changed to C/T (Y), and the C at position 17 was changed to T. Similarly, the forward primer was adapted by changing the G at position 12 to A (since it is present in all tested sequences except for /IRN/18/2010) and C at position 15 was changed to Y. There were fewer changes made in the reverse primer: T instead of G at position 8, and C instead of G at position 11 (Table 1).

The two assays were utilized together in a multiplex format for examining their ability to identify all the samples. The method was examined with all Israeli isolates from 1982-2014, and was shown that all samples including Neve Ur 2010 and Tira 2014, which were undetectable by the first assay (Table 2), were now detected.

Table 1: In-house designed primers and probes used in this work

First qPCR system	Sequence	Position in acc. KM921827
Forward primer	GTGGCAGTGAAG*CAC*GAGG	232
probe	FAM-TGGAC*AACA*CCACC*AAC*CCAACA*G-BHQ_	293
Reverse primer	GTAGCCAAC*AC*CGGTGTG	365
Second qPCR system		
Forward primer	GTGGCAGTGAAA^CAY^GAGG	232
probe	VIC-TGGAT^AACR^CCACY^AAT^CCAACG^G-BHQ_	293
Reverse primer	GTAGCCAAG^ACA^CGGTGTG	365

* – indicate nucleotides to be changed. ^ – indicate changed nucleotides.

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O/ISR/1/2013          CGCAGATTTAGAGGTGGCAGTGAAACACGAGGGGAACCTTACCTGGGTCCCGAATGGGGC 278
O/ISR/2/2013          CGCAGATTTAGAGGTGGCAGTGAAACACGAGGGGAACCTTACCTGGGTCCCGAATGGGGC 278
O/ISR/3/2013          CGCAGATTTAGAGGTGGCAGTGAAACACGAGGGGAACCTTACCTGGGTCCCGAATGGGGC 278
Madgel-Shams          CGCAGATTTAGAGGTGGCAGTGAAACACGAGGGGAACCTTACCTGGGTCCCGAATGGGGC 299
PAK10-2006            CGCAGATCTAGAGGTGGCAGTGAAACACGAGGGGAACCTTACCTGGGTCCCGAATGGGGC 300
PAK14-06              CGCAGATCTAGAGGTGGCAGTGAAACACGAGGGGAACCTTACCTGGGTCCCGAATGGGGC 278
PAK08-06              CGCAGATCTAGAGGTGGCAGTGAAACACGAGGGGAACCTTACCTGGGTCCCGAATGGGGC 278
JOR-06                CGCAGATCTAGAGGTGGCAGTGAAACATGAGGGGAGCCTTACCTGGGTCCCGAATGGGGC 278
BHU15-03              CGCAGATCTAGAGGTGGCAGTGAAACACGAGGGGAACCTTACCTGGGTCCCGAATGGGGC 278
NEP-03                CGCAGATCTAGAGGTGGCAGTGAAACACGAGGGGAACCTTACCTGGGTCCCGAATGGGGC 278
BHU49-03              CGCAGATCTAGAGGTGGCAGTGAAACACGAGGGGAACCTTACCTGGGTCCCGAATGGGGC 278
AFG-04                CGCAGATCTAGAGGTGGCAGTGAAACACGAGGGGAACCTTACCTGGGTCCCGAATGGGGC 278
AFG-03                CGCAGATCTAGAGGTGGCAGTGAAACACGAGGGGAACCTTACCTGGGTCCCGAATGGGGC 278
O/IRN/49/2009         CGCAGATTTAGAGGTGGCAGTGAAACACGAGGGGAACCTTACCTGGGTCCCGAATGGGGC 278
Jerico-2013-11        CGCAGATTTAGAGGTGGCAGTGAAACACGAGGGGAACCTCACCTGGGTCCCGAATGGGGC 278
O/PAT/13/2013         CGCAGATTTAGAGGTGGCAGTGAAACACGAGGGGAACCTCACCTGGGTCCCGAATGGGGC 278
O/PAT/14/2013         CGCAGATTTAGAGGTGGCAGTGAAACACGAGGGGAACCTCACCTGGGTCCCGAATGGGGC 278
O/TUR/12/2013         CGCAGATTTAGAGGTGGCAGTGAAACACGAGGGGAACCTTACCTGGGTCCCGAATGGGGC 278
O/TUR/38/2013         CGCAGATTTAGAGGTGGCAGTGAAACACGAGGGGAACCTTACCTGGGTCCCGAATGGGGC 278
O/TUR/37/2013         CGCAGATTTAGAGGTGGCAGTGAAACACGAGGGGAACCTTACCTGGGTCCCGAATGGGGC 278
O/TUR/27/2013         CGCAGATTTAGAGGTGGCAGTGAAACACGAGGGGAACCTTACCTGGGTCCCGAATGGGGC 278
O/IRN/18/2010         CGCAGATCTAGAGGTGGCAGTGAAACATGAAGGGAACCTTACCTGGGTACCCAATGGGGC 278
Forward primer                    GTGGCAGTGAAGCACGAGG
                                  ***** ***** **  * *
New                               GTGGCAGTGAAACAYGAGG

O/ISR/1/2013          GCCCGAGACAGCGTTGGACAACACCACCAATCCAACGGCTTACCACAAGGCACCACTCAC 338
O/ISR/2/2013          GCCCGAGACAGCGTTGGACAACACCACCAATCCAACGGCTTACCACAAGGCACCACTCAC 338
O/ISR/3/2013          GCCCGAGACAGCGTTGGACAACACCACCAATCCAACGGCTTACCACAAGGCACCACTCAC 338
Madgel-Shams          GCCCGAGACAGCGTTGGACAACACCACCAATCCAACGGCTTACCACAAGGCACCACTCAC 359
PAK10-2006            GCCCGAGACAGCGTTGGATAACACCACTAATCCAACGGCTTACCACAAGGCACCGCTCAC 360
PAK14-06              GCCCGAGACAGCGTTGGATAACACCACTAACCCAACGGCTTACCACAAGGCACCGCTCAC 338
PAK08-06              GCCCGAGACAGCGTTGGATAACACCACTAATCCAACGGCTTACCACAAGGCACCGCTCAC 338
JOR-06                GCCCGAGACAGCGTTGGATAACACCACTAATCCAACGGCTTACCACAAAGCACCGCTCAC 338
BHU15-03              GCCCGAGACAGCGTTGGATAACACCACTAATCCAACGGCTTACCACAAGGCACCGCTCAC 338
NEP-03                GCCCGAGACAGCGTTGGATAACACCACTAATCCAACGGCTTACCACAAGGCACCGCTCAC 338
BHU49-03              GCCCGAGACAGCGTTGGATAACACCACTAATCCAACGGCTTACCACAAGGCACCGCTCAC 338
AFG-04                GCCCGAGACAGCGTTGGATAACACCACTAATCCAACGGCTTACCACAAGGCACCGCTCAC 338
AFG-03                GCCCGAGACAGCGTTGGATAACACCACTAATCCAACGGCCTACCACAAGGCACCGCTCAC 338
O/IRN/49/2009         GCCCGAGACAGCGTTGGATAACACCACCAATCCAACGGCTTACCACAAGGCACCACTCAC 338
Jerico-2013-11        GCCCGAGACAGCGTTGGACAACGCCACCAATCCAACGGCTTACCACAAGGCACCACTCAC 338
O/PAT/13/2013         GCCCGAGACAGCGTTGGACAACGCCACCAATCCAACGGCTTACCACAAGGCACCACTCAC 338
O/PAT/14/2013         GCCCGAGACAGCGTTGGACAACGCCACCAATCCAACGGCTTACCACAAGGCACCACTCAC 338
O/TUR/12/2013         GCCCGAGACAGCGTTGGACAACACCACCAATCCAACGGCTTACCACAAGGCACCACTCAC 338
O/TUR/38/2013         GCCCGAGACAGCGTTGGACAACACCACCAATCCAACGGCTTACCACAAGGCACCACTCAC 338
O/TUR/37/2013         GCCCGAGACAGCGTTGGACAACACCACCAATCCAACGGCTTACCACAAGGCACCACTCAC 338
O/TUR/27/2013         GCCCGAGACAGCGTTGGACAACACCACCAATCCAACGGCTTACCACAAGGCACCACTCAC 338
O/IRN/18/2010         GCCTGAGAAGGCGTTGGACAACACCACCAATCCAACGGCTTACCACAAGGCACCGCTCAC 338
probe                              TGGACAACACCACCAACCCAACAG
                                   **** *** **** ** ***** *
New                                TGGATAACRCCACYAATCCAACGG

rfO/ISR/1/2013        CCGACTTGCCCTGCCTTACACGGCACCACACCGTGTCTTGGCTACCGTATACAACGGGAG 398
O/ISR/2/2013          CCGACTTGCCCTGCCTTACACGGCACCACACCGTGTCTTGGCTACCGTATACAACGGGAG 398
O/ISR/3/2013          CCGACTTGCCCTGCCTTACACGGCACCACACCGTGTCTTGGCTACCGTATACAACGGGAG 398
Madgel-Shams          CCGACTTGCCCTGCCTTACACGGCACCACACCGTGTCTTGGCTACCGTATACAACGGGAG 419
PAK10-2006            CCGACTTGCACTGCCTTACACGGCACCACACCGTGTCTTGGCTACCGTATACAACGGGAA 420
PAK14-06              CCGACTTGCACTGCCTTACACGGCACCACACCGAGTCTTGGCTACCGTATACAACGGGAA 398
PAK08-06              CCGACTTGCACTGCCTTACACGGCACCACACCGTGTCTTGGCTACCGTATACAACGGGAA 398
JOR-06                CCGACTTGCACTGCCTTACACGGCACCACACCGTGTCTTGGCTACCGTATACAACGGGAA 398
BHU15-03              CCGACTTGCACTGCCTTACACGGCACCACACCGTGTCTTGGCTACTGTTTACAACGGGAA 398
NEP-03                CCGACTTGCACTGCCTTACACGGCACCACACCGTGTCTTGGCTACTGTTTACAACGGGAA 398
BHU49-03              CCGACTTGCACTGCCTTACACGGCACCACACCGTGTCTTGGCTACTGTTTACAACGGGAA 398
AFG-04                CCGACTTGCACTGCCTTACACGGCACCACACCGTGTCTTGGCTACTGTTTACAACGGGAA 398
AFG-03                CCGACTTGCACTGCCTTACACGGCACCACACCGTGTCTTGGCTACTGTTTACAACGGGAA 398
O/IRN/49/2009         CCGACTTGCACTGCCTTACACGGCACCACACCGTGTCTTGGCTACCGTATACAACGGGAG 398
Jerico-2013-11        CCGACTTGCCCTGCCTTACACGGCACCACACCGTGTCTTGGCTACCGTATACAACGGGAG 398
O/PAT/13/2013         CCGACTTGCCCTGCCTTACACGGCACCACACCGTGTCTTGGCTACCGTATACAACGGGAG 398
O/PAT/14/2013         CCGACTTGCCCTGCCTTACACGGCACCACACCGTGTCTTGGCTACCGTATACAACGGGAG 398
O/TUR/12/2013         CCGACTTGCACTGCCTTACACGGCACCACACCGTGTCTTGGCTACCGTATACAACGGGAG 398
O/TUR/38/2013         CCGACTTGCACTGCCTTACACGGCACCACACCGTGTCTTGGCTACCGTATACAACGGGAG 398
O/TUR/37/2013         TCGACTTGCACTGCCTTACACGGCACCACACCGTGTCTTGGCTACCGTATACAACGGGAG 398
O/TUR/27/2013         CCGACTTGCACTGCCTTACACGGCACCACACCGTGTCTTGGCTACCGTATACAACGGGAG 398
O/IRN/18/2010         CCGACTTGCACTGCCTTACACGGCACCACACCGTGTCTTGGCTACCGTGTACAACGGGAA 398
reverse primer                                  CACACCGCGTGTTGGCTAC
                                                ******* ** ********
New                                             CACACCGTGTCTTGGCTAC
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Figure 1: ClustalW2 analysis of primers, Probes, and VP1 sequences of recent Israelis isolates. * – indicates the nucleotide changes in the new detection system. Accession no. are as published (Stram et al., 2011: Stram et al., 2015, submitted for publication)

Table 2: Comparison between the first, the improved O specific and the non-specific FMDV qRT-PCR assays. = The FMDV O specific assays were performed as multiplex reactions

Sample Name	First O Specific assay C_T	O Specific assay C_T	general FMDV assay C_T
Dalton1982	20.22	24.54	25.49
Gshur 2000	13.29	17.65	NT
Never Ur 2008	26.95	21.72	21.77
Natur 2008	23.88	18.58	17.75
Never Ur 2010	UD	17.75	NT
Manisa 2011	17.01	20.98	13.89
Gshur 2013	15.73	19.90	16.29
Jerico 2013	19.98	15.35	NT
Tira 2014	UD	14.28	NT
Dura-Daharya 2014	-	**28.12**	**25.2**
Negative	UD	UD	UD
A ISR 2009	UD	UD	UD

UD – undetected. NT – not tested.

To further validate the method, a comparison was made with a non-specific qPCR assay, able to detect any FMD virus regardless of theserotype, based on the conserved 3D region (16). It was noticed that the newly developed assay was in full agreement with the FMDV non-specific qPCR assay.

Furthermore, it was observed that several samples the cycle threshold (C_T) of both assays (O specific and non-specific) were highly similar. Dalton 1982 with C_T of 24.54 and 25.59 , Neve Ur 2008 with C_T 21.72 and 21.77 Nature 2008 with C_T of 18.58 and 17.71, respectively, were found to be similar (Table 2), although other samples differed in their C_T values. This validated the new assay as suitable to be used as a diagnosis tool for the isolates with the potential for detecting new incoming O serotype viruses.

To assess the specificity of the new method all seven FMDV serotypes (Table 3) were utilize in the assay. All serotypes except for O were negative. It is worth noticing that isolate C NEP 1/94 showed C_T value of 36, which is considered negative or at least significantly non-positive.

In order to demonstrate the practicality of the test, a tongue epithelial sample taken during an outbreak in the Palestinian Territories (Dura-Daharya 2014) was tested utilizing the recently established procedure in addition to the general qPCR assay. It was evident that both tests could detect and identify the virus responsible for the disease outbreak with C_T of 25.5 with the general FMDV test and C_T of 28.3 with FMDV O test (Table 3).

Like any other qPCR assays, the technique is highly sensitive to the template mutations particularly in the probe sequences. It was suggested that major outbreaks in the Middle East, including Israel, result from a newly arrived virus (15). Thus, there is always a chance, as remote as it is, of a virus with sufficient nucleotide changes in the primers and probe region arriving, which will escape detection.To illustrate the probability of such an occurrence, a system able to detect Middle Eastern O serotype (17) was examined by performing homology analysis using the probe and primers

Table 3: Specificity test of the new qPCR assay. Samples of all seven FMDV serotypes were tested by the newly developed qRT-PCR

Sample Name	C_T
A – Iraq 06	UD
A – Egp 13	UD
A – Kenya 11	UD
Asia 1 - Shamir	UD
C SRL 1/84	UD
C SAU 1/84	UD
C BHU 2 /94	UD
C PHI 11/89	UD
C NEP 1/94	36.05
SAT-1 Bot	UD
SAT-2 Zim	UD
SAT-3 Zim	UD

with the Israeli VP1 database. It was shown that in the 2013-14 isolates responsible for several outbreaks in the Palestinian Territories the A located at the 3' of the primer was changed to G. It was considered that this change could possibly hamper the ability of the assay to detect these viruses.

In summary, this work represents the development of a qRT-PCR assay that enables the detection of FMDV O serotype viruses found in past in the Middle East and provides a highlikelihood that it will be able to detect future introductions of O serotype viruses. It was already reported that the Middle East is a sensitive region for constant introduction of FMDV and particularly of O serotype. Therefore, it is paramount to have an assay available that is suitable for the task of detecting viruses residing in the area with the ability to identify future and new virus introductions, thus making it valuable to Middle Eastern laboratories engaged with FMDV.

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