Standardization of RT-PCR Protocol for Detection and Typing of Emerging Food and Mouth Disease Virus in Lebanon

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ABSTRACT

The applicability of developed RT-PCR protocols at OIE/FAO World Reference Laboratory in diagnostic of FMD is challenged by the continuous emergence of new lineage of isolates around the world, and specifically in the Eastern Mediterranean region where the genetic studies are very limited. The purpose of RT-PCR standardization is to amplify the conserved 5' UTR gene of FMD virus, using an application of variable RNA concentrations and cycling conditions on two tongue-epithelial tissue samples, collected from cows (FMD1 and FMD2) provided by the Lebanese Ministry of Agriculture, to optimize the FMD typing, and to apply the established protocols on other suspected cases of FMD. The RNA amount was set in a volume of 4µL at 20 ng/µL and the optimal RT-PCR cycling condition was established at 50°C for 45 minutes, followed by 15 minutes at 95°C, then by 30 cycles of 94°C for 1 minute, a lower temperature of 55°C for 1 minute, then a higher temperature of 72°C for 2 minutes, followed by one cycle at 72°C for 7 minutes. The optimized amplification resulted in 328 bp band from the two field samples (FMD1 and FMD2). The optimized RT-PCR protocol applied on four additional field samples collected from two lactating cows, revealing two additional positive isolates of FMD (FMD3 and FMD4). The RT-PCR was used in typing of the positive FMD samples with different sets of primers specific for various FMD types, revealing SAT2 type in samples FMD1,

FMD3 and FMD4, and failed in typing the FMD2 sample. The importance of the optimization of RT-PCR, in uncovering the presence of new types of FMD in livestock of this region, and in establishment of future FMD control programs, is discussed.

INTRODUCTION

Reverse Transcription Polymerase Chain Reaction (RT-PCR) is presently adopted as an indispensable tool and a golden standard for diagnosis of Foot and Mouth Disease (FMD), providing high sensitivity and specificity in detection and serotyping of FMD isolates.[1-3] However, the applicability of RT-PCR in diagnosis and control of this disease is constantly challenged by the emergence of new strains, specifically in the Middle Eastern region and other developing countries.[2,3] Periodically, new genetic lineages appear to spread in a westward direction from the Indian subcontinent through Pakistan–Afghanistan–Iran–Turkey, or via importation of animals or animal products into the Gulf States and Egypt, specifically of the SAT2, O, and other subtypes.[3-6] The precise origin of these FMD epidemics is still unknown due to absence of reliable regional epidemiology; moreover, only limited genetic studies of FMDV from countries of the Middle East have been reported.[7-11] The primer sets used in RT-PCR standardization in other parts of the world, including those developed in reference labs of OIE and FAO; such primers might not be applicable for diagnosis of newly emerging serotype/subtypes or even topotypes that include genetically distinct strains, within a serotype that are present within a particular geography.[1,5,7]

The applicability of developed RT-PCR in diagnosis of FMD was further hindered by the fact that detection of all seven serotypes of the virus, using specific recommended primers by the RT-PCR protocols of OIE/FAO World Reference Laboratory for FMD(WRL) in Pirbright, was not fully adequate for the serotyping of suspensions prepared from clinical samples of epithelium, and sometimes required previous cell culturing of specific isolates in order to improve the protocol sensitivity in serotyping.[3] Moreover, it is important to recognize that the reproducibility of RT-PCR protocols is also related to the instruments and reagents brand that were used during the standardization protocols.[12]

Accordingly, it is essential to standardize the RT-PCR protocol, using local unidentified serotypes of field isolates, to help in future implementation of proper epidemiology of FMD in endemic areas of the world, and in evaluation of vaccine protection implemented in developing countries,[5] where the genetic studies are very limited. In this study, we aimed at the standardization of the RT-PCR protocol for diagnosis of newly emerging FMD isolates in the Eastern Mediterranean region of Lebanon, for the purpose of making it available for future investigations by different workers in this region, and other parts of the world, where FMD is endemic.

The RT-PCR standardization includes the use of two field bovine samples (FMD1 and FMD2) and variable viral RNA concentrations and cycling conditions. The standardized protocol will be applied on other uncharacterized four field samples, followed by typing of the positives using also the optimized typing protocol.

MATERIALS AND METHODS

FMD Samples

A total of six dairy samples were collected in transport medium, prepared according to WHO protocol [WHO, 2006]. Two of the six samples (FMD 1 and FMD2) were of tongue-epithelial tissue nature collected from FMD suspected cows, and delivered to us by the Ministry of Agriculture in Lebanon. Two other dairy samples (FMD3 and FMD4) were collected from a farm in Mount Lebanon, showing typical FMD lesions in its beef and dairy herds. The FMD3 and FMD4 tongue-scraping samples were collected from two lactating cows that were vaccinated 7 months prior to observation of the FMD-lesions and sampling. An additional two tongue-scraping samples (FMD5

Table 1: *List of primers for the seven FMD types and the expected size of PCR amplicon for each type.*

Type	Primers sequence 5'-3'	Primer concentration used in 50 µL RT-PCR mixture (pmol)	Expected size of PCR amplificons (bp)	References
O	F: ACCAACCTCCTTGATGTGGCT	20	1301	(Reid et al., 2000)
	R: GACATGTCCTCCTGCATCTG	20		
A	F: TACCAAATTACACACGGGAA	20	863–866	
	R: GACATGTCCTCCTGCATCTG	20		
C	F: TACAGGGATGGGTCTGTGTGTACC	20	877–833	
	R: GACATGTCCTCCTGCATCTG	20		
SAT1	F: AGGATTGCCAGTGAGACCCACAT	15	246	(Fernandez et al., 2008)
	F:AGGAT(T/C)GC(A/C)AG(T/C)GAGAC(A/G)CACAT	90		
	F: AGGATTGCTAGTGAGAC(A/C)CACAT	30		
	F: AGGATTGCAAGCGAGACCCACAT	30		
	R: GAAGGGCCCAGGGTTGGACTC	30		
SAT2	F: GGCGTTGAAAAACAACT(G/C)TG	30	75	
	F: GGCGTCGCGAAACAGCTTTT	15		
	F: GG(C/T)GT(C/T)GA(A/G)AAACA(A/G)(C/T)TGTG	30		
	R: GAAGGGCCCAGGGTTGGACTC	30		
SAT3	F: TTCGGAAGATTGTTGTGTG	15	201	
	F: TTCGG(T/G)AGA(C/T)TGTTGTGTG	15		
	F: TTCGGTAGGCTGTTGTGTG	15		
	F: TTCGGGAGACTGTTGTGCG	15		
	F: TTCGG(T/G)AG(A/G)(C/T)TGTTGTGTGA	45		
	R: GAAGGGCCCAGGGTTGGACTC	30		
Asia 1	F: GACACCACTCAGGACCGCCG	30	296	(Reid et al., 1999)
	F: GACACCACCCAGGACCGCCG	30		
	F: GACACCACACAAGACCGCCG	30		
	F: GACACGACTCAGAACCGCCG	30		
	R: AGCTTGTACCAGGGTTTGGC	30		

and FMD6) were collected from a beef farm in an East suburban farm of Beirut capital that had around 300 beef heads, not vaccinated against FMD, and imported from Europe. All FMD-suspected animals showed vesicular lesions on the tongue, hoofs and udder (in case of females).

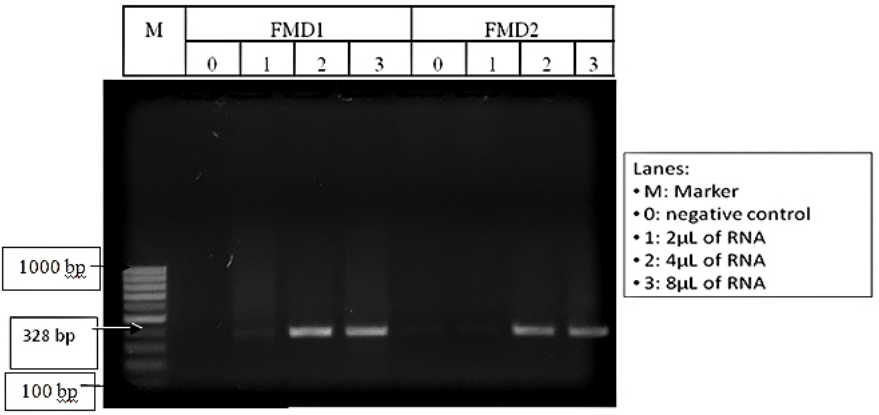
Collected samples were homogenized in Transport Medium, using PRO 300D homogenizer (PRO Scientific Inc., Oxford, CT06478, USA), and subjected to a three-time freezing and thawing procedure, in an attempt to release the viral particles from the host cell.

Standardization of RT-PCR

The Amplification of 5' UTR Conserved Gene of Field Isolates FMD1 and FMD2

The RNA of FMD1 and FMD2 samples was extracted as per the recommendation of the QIAamp viral RNA minikit procedure. The extracted RNA was used for standardization of RT-PCR amplification of the 5'

Figure 1: *Standardization of RT-PCR for 5' UTR gene at higher temperature of 50°C for reverse transcription and at varied RNA concentrations of Food and Mouth Disease Viruses in FMD1 and FMD2 samples. The gel is divided into three major columns namely, M for 100 bp marker,FMD1 and FMD2 each containing lanes 0, 1, 2, and 3 for respective inclusions in the PCR mixture of 0, 2, 4, and 8 µl of 20 ng/µL of viral RNA. The first cycling condition of this experiment is detailed under section B.1.*



untranslated universal region (5' UTR) gene, using variable RNA concentrations and cycling conditions. Volumes of 2, 4, and 8 µL of 20 ng/µL RNA extract of the FMD1 and FMD1 homogenate samples were individually added to a 50µl reaction mixture containing 4 pmol of each of the forward 1F (5'- GCCTGGTCTTTCCAGGTCT- 3') and reverse 1R (5'- CCAGTCCCCTTCTCAGATC-3') primers, 2µl of OneStep RT-PCR Enzyme Mix, 10µl OneStep RT-PCR buffer, 2µl of 10mM dNTP mix. The volume was completed to 50µl with nuclease-free water.

Two cycling conditions were used in the standardization protocol. First, The RT-PCR cycling conditions were set as follows: 45 minutes at 37°C, followed by 5 minutes at 94°C, then by 30 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes. A final extension step was performed at 72°C for 7 minutes. Second, The RT-PCR cycling conditions were changed to 45 minutes at 50°C, followed by 15 minutes at 95°C, then by 30 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes. A final extension step was performed at 72°C for 7 minutes.

*Typing of FMD1 and FMD2 isolate***s**

Typing of the FMD isolates in the two field samples was performed using a set of primers specific for O, A, C, Asia 1, SAT1, SAT2 and SAT3 (Table 1). The SAT multimix was a mixture of primers of SAT 1, 2 and 3 that were described in Table 1.

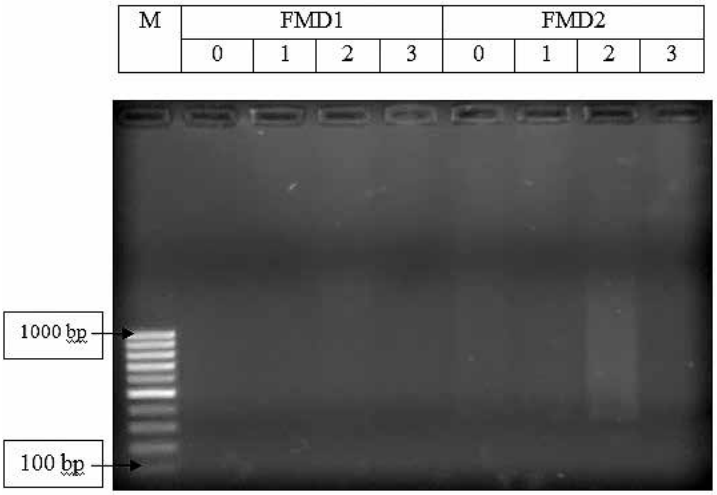
Typing for O, A, C

An amount of 4 µL of 20ng/µL of extracted RNA was added to the RT-PCR mixture consisting of 2µL of OneStep RT-PCR Enzyme Mix, 10µL OneStep RT-PCR buffer, and 2µL of 10mM dNTP mix. The primers concentrations are presented in Table 1. The volume is completed to 50µL with nuclease-free water. The RT-PCR cycling conditions were 45 minutes at 50°C, followed by 15 minutes at 95°C, 30 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes. A final extension step was performed at72°C for 7 minutes. The resulting amplicons were visualized following agarose-gel electrophoresis and EZvision staining under UV-light.

Typing for SAT 1, 2, 3 and Asia 1

The primers used for each of the types are indicated in Table 1.The same dNTP, enzyme mix, buffer concentrations, RNA concentration were used as those listed above

Figure 2: *Standardization of RT-PCR for 5' UTR gene at lower temperature of 37°C for reverse transcription and at varied RNA concentrations of Food and Mouth Disease Viruses in FMD 1 and FMD2 samples. The Gel is divided into three major columns namely, M for 100 bp marker, FMD 1 and FMD2 columns each containing lanes 0, 1, 2, and 3 for respective inclusions in the PCR mixture of 0, 2, 4, and 8 µl of 20ng/µL of viral RNA. The second cycling condition of this experiment is detailed under section B.1.*



for O, A and C typing. However, for SAT1, SAT2 and SAT3 the cycling conditions were as follows: 45 minutes at 50°C, 15 minutes at 95°C, 35 cycles of 0.15 minute at 95°C, 60°C for 0.3 minutes,72°C for 0.3 minutes, and a final extension step of 7 minutes at 72°C. As for Asia 1, the cycling conditions were 45 minutes at 50°C, 94°C for15 minutes, 20 cycles of 94°C for 1 minute, 58°C for 1 minute, and 72°C for 2 minutes.13 A final extension step was performed at 72°C for 7 minutes. The expected amplicon for each type is indicated in Table 1.

Evaluation of the Standardized RT-PCR Protocols in Screening and Typing of Suspected FMD Viruses inFMD3, FMD4, FMD5 and FMD6 Samples

Screening for conserved 5' UTR FMD gene

The evaluation of the standardized protocol for 5'UTR FMD gene application, was accomplished by its application on suspected FMD viruses in the four samples. The RNA was extracted as per the recommendation of QIAamp viral RNA minikit procedure. A volume of 4 µL of 20ng/µL RNA extract of each sample was individually added to a 50µl reaction mixture, containing 4pmol of each of the forward 1F (5'- GCCTG-GTCTTTCCAGGTCT-3') and reverse 1R (5'-CCAGTCCCCTTCT-CAGATC-3') primers, 2µl of OneStep RT-PCR Enzyme Mix, 10µl OneStep RT-PCR buffer, and 2µl of 10mM dNTP mix. The volume was completed to 50µl with nuclease-free water. The RT-PCR cycling conditions were set as follows: 45 minutes at 50°C, followed by 15 minutes at 95°C, 30 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes. A final extension step was performed at 72°C for 7 minutesTyping

Typing was performed on the FMD3 and FMD4 samples that were positive for conserved gene 5' UTR of FMD. Each primer set targets a specific region of the VP1 gene. The same standardized procedures and cycling conditions that were used for typing of FMD1 and FMD2 were also evaluated by their application on the genomes of the viruses in FMD3 and FMD4 samples.

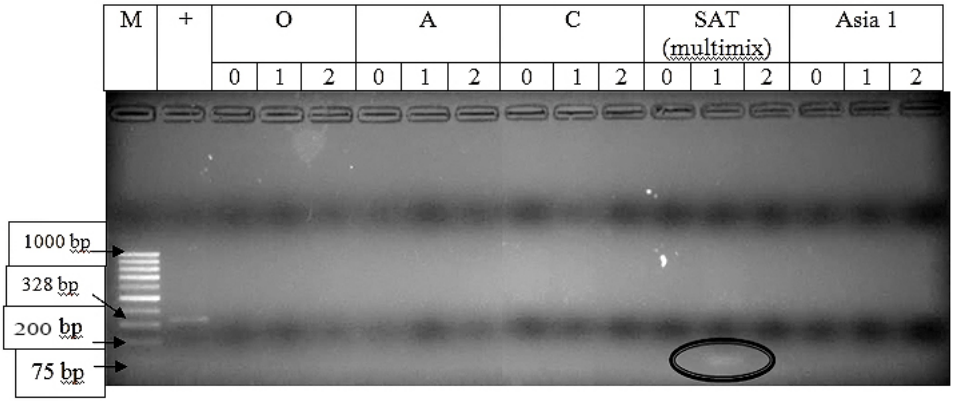
RESULTS AND DISCUSSION

Standardization of RT-PCR Protocols for 5' UTR gene Amplification and for typing of FMD Viruses

Standardization of 5'UTR Gene Amplification of FMD1 and FMD2 Isolates

The wo samples provided from the Ministry of Agriculture were scraping of dairy cow-tongues, suspected as FMD positive. These samples were used for the RT-PCR standardization protocol to amplify the conserved

Figure 3: *Standardized RT-PCR for typing of Foot and Mouth Disease Virus in FMD1 and FMD2 samples at varied cycling conditions for the different types. The Gel image is divided into seven major columns namely, M for 100 bp marker, (+) for the positive control for the 5' UTR gene amplification of FMD1 (standardized in Fig.1 of lanes 2 and 3), columns O, A, C, SAT, Asia1 for respective types of FMD and each containing three lanes namely, 0, 1, and 2, where 'O' corresponds to negative RT-PCR mixture lacking RNA, '1' corresponds for inclusions of viral RNA in FMD1 sample, and '2' corresponds for inclusions of viral RNA in FMD2 sample. The cycling conditions used in this experiment for each type of FMD are described under section B.2.a and B.2.b.*



5'UTRgene.

The F1/R1 primer set was used for primary detection of FMD virus in the samples. An amplicon of the expected size (328bp) was considered a positive result.1 A band with this expected size (328bp) was obtained from FMD1 and FMD2 samples at 4 and 8 µl of the 20 ng/µl RNA concentration and using the RT-PCR cycling conditions of 45 minutes at 50°C, followed by 15 minutes at 95°C, then by 30 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes, and one cycle at 72°C for 7 minutes (Figure 1). The use of 37°C, as indicated by Reid et al. in 1999,1 instead of 50°C at the reverse transcription step failed to amplify the conserved generic gene of FMD virus (Fig. 2). This could be due to the use of Promega RT-PCT kit in Reid's work13 instead of Qiagen kit that necessitates the use of 50°C temperature for reverse transcription. The F1/R1 primers were able to anneal to the generic 5'UTR gene of the suspected FMD virus samples and were thus considered FMD positive.13 Accordingly, the 4 µL of 20 ng/µL RNA and the successful cycling conditions including 50°C for reverse transcription were used in all RT-PCR runs that followed.

Typing of FMD1 and FMD2 Isolates

The RT-PCR technique was applied on FMD1 and FMD2 isolates using different primer sets specific for each type (Table 1). A band was obtained at 75 bp for the RT-PCR mixture of FMD1 genome, containing the SAT1/SAT2/SAT3 primer multimix which indicated that FMD1 is of type SAT2 (Fig.3).13 All the other RT-PCR mixtures failed to give a band, which means that primers failed to anneal to the part of the VP1 gene specific for each type. The reason for FMD2 typing failure could be the low detection limits of the SAT2 primers on the FMD2 sample containing a different variant that needs a construction of specific primers for it; in this context, enhanced detection limit of the SAT2 primers was reported when the virus was propagated in cell culture as compared to the initial sample.[13,14] Future investigations will attempt the propagation of these viruses in cell-cultures before applying the RT-PCR on the collected tissue.

Evaluation of the Standardized RT-PCR Protocols in Screening and Typing of Suspected FMD Viruses in FMD3, FMD4,

Figure 4: *Evaluation of standardized RT-PCR of 5'UTR gene in Fig 1, in its application on genome of FMD3, FMD4, FMD5 and FMD6 samples. The Gel image is divided into seven major columns namely M column for 100 bp marker, '+' for positive control of 5'UTRgene amplification FMD1 (standardized in Fig 1 of lanes 2 and 3), '-' for negative control (RT-PCR mixture lacking RNA of FMD1 sample), FMD3, FMD4, FMD5 and FMD6 columns for respective viruses in the four unknown samples.*

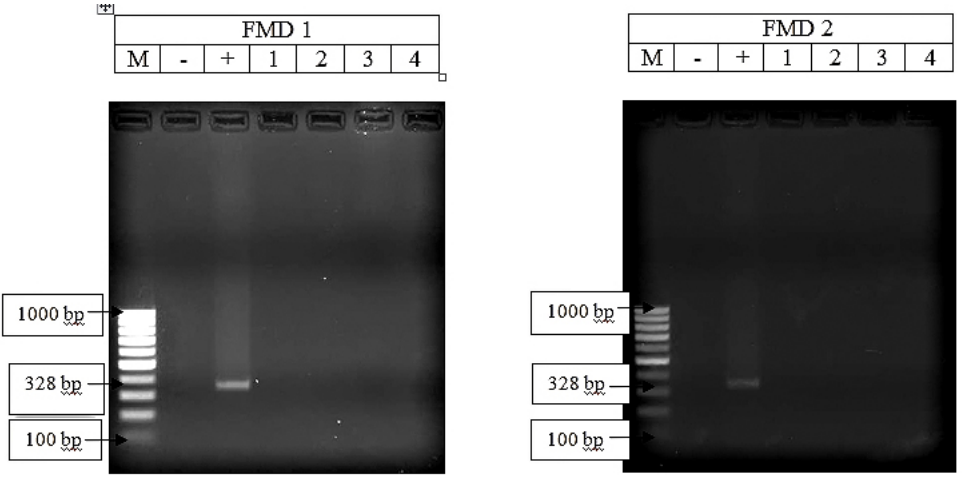


Figure 5: *Evaluation of standardized RT-PCR for typing of food and Mouth Disease Viruses present in FMD3 and FMD4 samples. The Gel image has parts "I" and "II", divided into major columns namely, 'M' for 100bp marker, 'G' for 5'UTR of conserved gene of FMD, 'A' and 'O' columns for the 'A' and 'O' types of FMD, 'SAT', 'Asia1' and 'C' columns for the three other types of FMD. The lanes 0, 1, 2, in Part I correspond respectively to negative (no RNA in the PCR mixture), and to genomes of FMD3 and FMD4 viruses. The lane 1 under 5'UTR of part II is for genome of FMD4; the lanes 0, 1 and 2 under each type of SAT, Asia1 and C in part II of the image are for respective negative, genomes of FMD3 and FMD4. The SAT multimix column includes PCR mixtures with primers for SAT1, 2 and 3 (Table 1).*

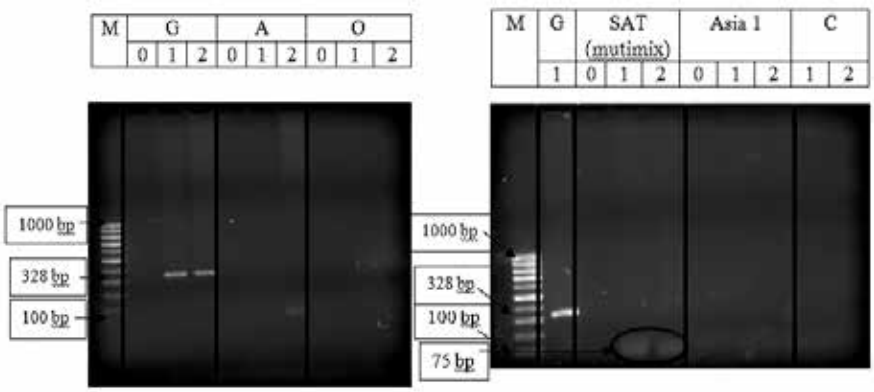
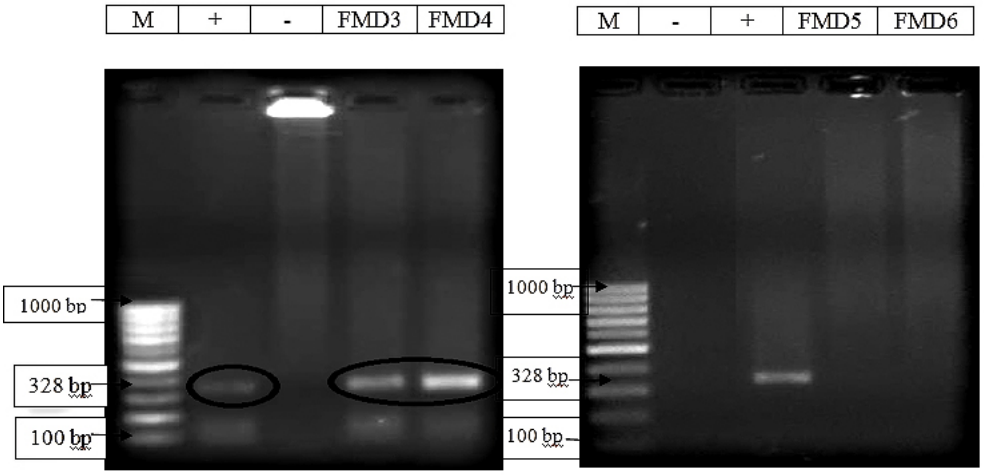


Figure 6



FMD5 and FMD6 Samples Screening for conserved 5'UTR of FMD gene

The standardized procedure was applied on FMD3, FMD4, FMD5, and FMD6 isolates using the F1/R1 primer set for generic 5' UTR gene (Fig. 4). RT-PCR amplification of FMD3 and FMD4 isolates resulted in a band of 328 bp which indicated that they are FMDV isolates.1 However, RT-PCR amplification of FMD5 and FMD6 did not result in any band, which could be due to FMD absence from FMD5 and FMD6 samples, F1/R1 primer, or that the lesions on the animals might not be caused by the FMD virus but to an etiological agent that resulted in similar clinical signs in infected animals.[15,16] This requires further differential diagnosis to identify the causative agent, with future focus on differentiation of FMD virus from vesicular stomatitis virus, or to sequence the whole viral genome to reveal possible mutations that could have occurred at the primer annealing site, then design primers to target these sites.

Typing of FMD3 and FMD4 Isolates

Typing procedure of RT-PCR was performed on the FMD3 and FMD4 isolates that were FMD positive. RT-PCR technique was performed using different primer sets specific to each type on FMD3 and FMD4 isolates (Table 1). A band was obtained at 75 bp for FMD3 and FMD4, using a PCR mixture containing the SAT1/SAT2/SAT3 primer multimix which indicated that FMD3 and FMD4 are of type SAT2 (Fig.5).[13]

CONCLUSIONS

The optimal RT-PCR conditions to amplify the conserved 5' UTR gene of FMD virus included the use of 4µL of 20 ng/µL RNA concentration extracted from the tongue scraping in 50µL RT-PCR mixture with the following cycling conditions: 45 minutes at 50°C, followed by 15 minutes at 95°C, then by 30 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes, one cycle at 72°C for 7 minutes. Four out of the six bovine lingual tissue samples were positive for FMD as revealed by RT-PCR. Typing using specific primer sets for each type revealed that positive samples were of type SAT2, which was not included in the two most commonly used vaccines in Lebanon, namely Cinko (containing subtypes A Iran 2005, O Manisa, O Panasia2, Asia-1 produced in BHK-21 cells) and Aftovaxpur (Merial) (containing O Manisa, O-3039 Air05, and Asia-1). This explains the failure of these vaccines to protect cattle against the local FMD isolates.

It is recommended, in the future, to conduct continuous annual screening for FMD viruses at the national level to reveal the most prevalent FMD types, so as to tailor the manufacturing of vaccines according to

the nature of the prevalent FMD strains of Lebanon, to reach to a better protection that can improve the dairy and beef production, and consequently help in improving the food security of this country.

REFERENCES

1. Reid SM, Hutchings GH, Ferris NP, De Clercq K. Diagnosis of foot-and-mouth disease by RT-PCR: evaluation of primers for serotypic characterisation of viral RNA in clinical samples. *J Virol Methods.* 1999:83:113-123.
2. Hoffmann B, Beer M, Reid SM, Mertens P, Oura C, van Rijn PA, Slomka MJ, Banks J. A review of RT-PCR technologies used in veterinary virology and disease control: sensitive and specific diagnosis of five livestock diseases notifiable to the World Organization for Animal Health. *Vet Microbiol.* 2009:139:1-23.
3. Longjam N, Deb R, Sarmah AK, Tayo T, Awachat VB, Saxena VK. A brief review on diagnosis of Foot-and-Mouth disease of livestock: Conventional to molecular tools. *Vet Med Int.* 2011. doi:10.4061/2011/905768.
4. Valarcher JF, Leforban Y, Rweyemamu M, Roeder PL, Gerbier G, Mackay DKJ, Sumption KJ, Paton DJ, Knowles NJ. Incursions of foot-and-mouth disease in Europe between 1985 and 2006. *Transbound Emerg Dis*. 2008:55(1): 14-34. doi: 10.1111/j.1865-1682.2007.01010.x.
5. Jamal SM, Ferrari G, Ahmed S, Normann P, Belsham GJ. Molecular characterization of serotype Asia-1 foot-and-mouth disease viruses in Pakistan and Afghanistan; emergence of a new genetic Group and evidence for a novel recombinant virus. *Infect Genet Evol*. 2011:11:2049–2062.
6. Michieli G. Foot and mouth disease, Egypt (OIE, March 14 2012): 13 New SAT2 FMD virus outbreaks. 2012. Available: http://www.flutrackers.com/forum/showthread.php?p=446713
7. Knowles NJ, Samuel AR Molecular epidemiology of foot-and-mouth disease virus. *Virus Res*. 2003:91:65-80.
8. Klein J, Hussain M, Ahmad M, Normann P, Afzal M, Alexandersen S. Genetic characterization of the recent foot-and-mouth disease virus subtype A/IRN/2005. *Virol J.* 2007:4:122. doi:10.1186/1743-422X-4-122.
9. Klein J, Hussain M, Ahmad M, Afzal M, Alexandersen S. Epidemiology of Buffalo colony. *Virol J.* 2008:5: 53. doi:10.1186/1743-422X-5-53.
10. Schumann KR, Knowles NJ, Davies PR, Midgley RJ, Valarcher JF, Raoufi AQ, McKenna TS, Hurtle W, Burans JP, Martin BM, Rodriguez LL, Beckham TR. Genetic characterization and molecular epidemiology of foot-andmouth disease viruses isolated from Afghanistan in 2003–2005. *Virus Genes.* 2008:36: 401–413.
11. Waheed U, Parida S, Khan QM, Hussain H, Ebert K, Wadsworth J, Reid SM, Hutchings GH, Mahapatra M, King DP, Paton DJ, Knowles NJ. Molecular characterisation of foot-and-mouth disease viruses from Pakistan, 2005–2008. *Transbound Emerg Dis.* 2011:58:166–172.
12. Grunenwald H. Optmization of Polymerase Chain Reaction. In Bartlett JMS, Stirling D, editors. *Methods in Molecular Biology*, second edition. Humana Press, 999 Riverview Drive, Suite 208, Totowa, USA. 2003:89-99.
13. Reid SM, Ferris NP, Hutchings GH, Samuel AR, Knowles NJ. Primary diagnosis of foot-and-mouth disease by reverse transcription polymerase chain reaction. *J Virol Methods*. 2000:89:167-176.
14. King DP, Burman A, Gold S, Shaw AE, Jackson T, Ferris NP. Integrin sub-unit expression in cell cultures used for the diagnosis of foot-and-mouth disease. *Vet Immunol Immunopathol.* 2011:140:259-265.
15. Fernandez J, Aguero M, Romero L, Sanchez C, Belak S, Arias M, Sanchez-Vizcaino JM. Rapid and differential diagnosis of foot-and-mouth disease, swine vesicular disease, and vesicular stomatitis by a new multiplex RT-PCR assay. *J Virol Methods.* 2008:147:301-311.
16. Teifke JP, Breithaupt A, Haas B. Foot-and-mouth disease and its differential diagnoses. Tierarztliche Praxis Ausgabe G, Grosstiere/Nutztiere. 2012:40:225-237.