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Article

Using FTA Cards as a Rapid and Field Tool for Detection and Serotyping of Foot-and-mouth Disease Virus in Libya

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Simple Summary: Foot and Mouth Disease (FMD) is highly contagious viral disease affecting cloven-hoofed domestic and wild animals. FMD can lead to high economic losses to farmers due to decrease in animals' production and high mortality in young animals. Rapid and accurate diagnosis of FMD outbreak is the first and very important step in order to control and preventing further spread of the FMD virus. As no protection between different FMD serotypes, hence, particular vaccines against the circulating FMD serotype is crucial step in the control of the disease. Many countries especially developing countries facing difficulties in the diagnosis and sending samples to Reference laboratories to get final diagnosis. FTA cards can be an alternative and cheap method to send clinical samples at room temperature with ordinary courier.

Abstract: Foot and Mouth Disease (FMD) is a viral disease, widespread and highly contagious affecting mainly cloven-hoofed domestic and wild animals. FMD can lead to high economic losses due to reduction in animals' production such as drop of milk production, loss of body weight and high mortality rate in young ruminants. Sixteen blood samples, oral swabs, and epithelial tissues were collected from animals showing typical clinical signs of FMD during the last FMD outbreak in Libya in 2018-2019. Blood samples, oral swabs, and mouth and tongue epithelial tissues impressions on Flinders Technology Associates (FTA) cards were shipped to the FMD Reference laboratory in Brescia, Italy, and tested for the detection of FMD viruses. Nucleic acids were extracted from the FTA cards and molecular testing of the examined FTA cards confirmed that the FMD virus circulating in Libya was serotype O. Sequencing analysis of the FMD virus VP1 gene confirmed that FMD virus strain was serotype O/East Africa 3 (O/EA-3) topotype. The phylogenetic of the VP-1 gene of the FMD virus showed very high nucleotide identity (99.8%) between the virus circulating in Libya and the Algerian FMD virus strains isolated in Algeria on 2018 and 2019.

Keywords: FTA cards; foot-and-mouth disease; RT-PCR; field; sequence

1. Introduction

Foot-and-mouth disease (FMD) is a viral disease, widespread and highly contagious transboundary disease that can be counted as one of the most economically important devastating disease of livestock due to the diversity of the affected animal species, rapid spread between and within geographic regions, and its control difficulties. It affects almost all wild and domesticated cloven-hoofed mammals [1].

FMD virus is non-enveloped virus, positive-sense, single-stranded RNA genome and belongs to the genus Aphthovirus of the viral family Picornaviridae [1]. FMD virus has seven serotypes that are immunologically distinct and are known to depend on the fact that there is no cross protection between these seven serotypes [2]. In addition, each FMD

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virus serotype has a number of genetic and antigenic variants with different degrees of virulence known as topotypes [3].

FMD virus has four structural proteins known as VP1, VP2, VP3 and VP4. VP1, highly variable protein, has an important role in virus attachment and entry to the host cell, protective immunity by inducing neutralizing antibodies, and known for serotype specificity. Sequencing of VP1 provides practical way to distinguish between different FMD virus lineages and topotypes. VP1 Sequence data for serotype O showed that this virus can be grouped into 11 topotypes known as Euro-SA for Europe-South America topotype, ME-SA for Middle East-South Asia topotype, SEA for Southeast Asia topotype, CHY for Cathay topotype, WA for West Africa topotype, EA-1 for East Africa 1 topotype, EA-2 for East Africa 2 topotype, EA-3 for East Africa 3 topotype, EA-4 for East Africa 4 topotype, ISA-1 for Indonesia-1 topotype, and ISA-2 for Indonesia-2 topotype [4,5].

However, as there is no cross-protection between FMDV serotypes due to the significant genetic variation observed between FMDV genomes, this lead to the necessity for rapid and accurate identification of the circulating FMD virus serotype for vaccine matching process with strains of the same serotype as the field strain [6].

There are many obstacles and difficulties in Libya regarding shipping samples to international Reference laboratories. Hence, using Flinders Technology Associates (FTA) cards could be as an alternative and practical way to store and send clinical samples. FTA cards were created as a substitute for transporting and storing infected samples for molecular diagnostics. FTA cards are cotton-based cellulose cards with chemicals that burst cells, denature proteins, and protect nucleic acids, resulting in a sample suitable for molecular identification while avoiding disease contamination. They are a quick and easy way to get samples from the field to the laboratory for pathogen detection or biological testing. Cell membranes and organelles on FTA cards are lysed and the nucleic acids are released. The nucleic acids remain immobilized and are preserved for long time at room temperature. Moreover, using FTA cards, with inactivated genetic materials of microorganisms, can be easily shipped to any laboratory easily, cheap and without the need of the dry ice. Captured nucleic acids on FTA cards can be extracted and undergoes molecular diagnostics tests [7].

The aim of this study was to detect FMD virus in Libya using FTA card as a field tool and to further identify the FMDV serotyping and topotyping from the genetic material isolated from the FTA card.

2. Materials and Methods

2.1. Swab samples

Swabs were collected from the oral cavity of animals showing typical clinical signs of FMD by swabbing the oral mucosa and the tongue and putting the swabs into a 2-ml of sterile phosphate-buffered saline (PBS). The swabs were immediately kept on ice and then stored at -80°C for further processing.

2.2. Epithelial tissue

The tissue selected for sampling was the affected epithelial tissue from the mouth and the tongue of animals showing typical clinical signs of FMD. At least one gram of the epithelium was placed in a transfer medium of PBS or equal parts of a glycerol-phosphate solution with a pH of 7.2-7.6, and all samples were immediately kept on ice and then stored at -80°C for further processing.

2.3. Blood samples

EDTA-stabilized blood samples were collected from animals showing typical clinical signs of FMD. Minimum of 10 mL of blood sample was collected from each animal, placed on ice and then stored at -80°C for further processing.

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2.4. FTA cards

Sixteen samples including swabs from the mouth and tongue, epithelial tissues and blood samples from animals with clinical FMD symptoms (Cattle and sheep) were collected (Table 1). Samples were collected from animals in Libya from three different cities of the western Libya including Tripoli, Tajoura and Misrata. FTA cards were labeled and coded for each of the above mentioned samples (Figure 1). Impressions of the epithelial tissues, swabs and drops of blood samples on FTA cards were left to dry, and then prepared to be shipped by currier to the Reference laboratory (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER)) in Brescia, Italy.

Table 1. Details of all samples collected on FTA cards.

Sample	Host	Lesion age	Type of sample	City	Vaccinated	Temp.	Age	Collection date
1	Cattle	3 Days	Epithelial tissue	Misrata	No	39.5	3 Y	May 2019
2	Cattle	2 Days	Epithelial tissue	Misrata	No	39.5	3 Y	May 2019
3	Cattle	2 Days	Epithelial tissue	Misrata	No	39.5	3 Y	May 2019
4	Cattle	3 Days	Epithelial tissue	Misrata	No	N/A	N/A	May 2019
5	Cattle	2 Days	Epithelial tissue	Misrata	No	N/A	N/A	May 2019
6	Cattle	2 Days	Epithelial tissue	Misrata	No	N/A	N/A	May 2019
7	Cattle	2 Days	Oral swab	Misrata	No	39.5	3 Y	May 2019
8	Cattle	2 Days	Whole blood	Misrata	No	N/A	N/A	May 2019
9	Cattle	3 Days	Epithelial tissue	Tajoura	No	N/A	2 Y	May 2019
10	Cattle	3 Days	Whole blood	Tajoura	No	N/A	N/A	May 2019
11	Sheep	-	Oral swab	Tajoura	No	N/A	N/A	October 2018
12	Sheep	-	Oral swab	Tajoura	No	N/A	N/A	October 2018
13	Sheep	-	Oral swab	Tripoli	No	N/A	N/A	October 2018
14	Sheep	-	Oral swab	Tripoli	No	N/A	N/A	October 2018
15	Sheep	-	Oral swab	Tripoli	No	N/A	N/A	October 2018
16	Sheep	N/A	Epithelial tissue	Tripoli	No	N/A	N/A	October 2018

N/A: Not available.

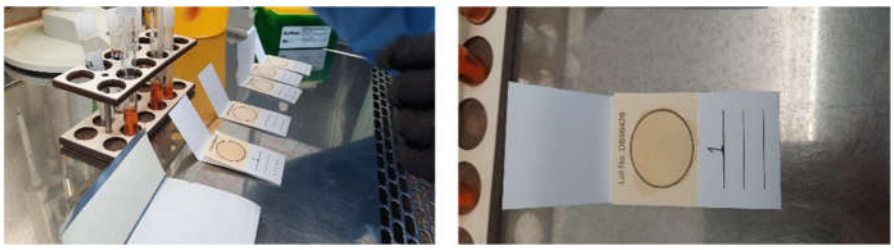

Figure 1. Preparing of samples on FTA cards.

2.5. RNA extraction

The method used to extract RNA from FTA cards consisted in a simple elution according to Muthukrishnan et al. [8], briefly: six sections of 5 mm in diameter were removed from each FTA card using a disposable biopsy punch (Kai industries). The six paper disks were divided in two 1.5 ml test tubes (three in each tube), 500 µL of minimum essential medium (MEM) was added and the tubes were incubated overnight at +4°C. For each sample the RNA was extracted from 140 µL of the overnight elution in MEM from one of the two duplicate tubes by using the QIAamp viral RNA extraction mini Kit (Qiagen).

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2.6. Real-Time RT-PCR

Two Real-time RT-PCR tests were performed on the extracted RNA: the first one, targeting the 3D gene of FMDV genome, that is able to detect all FMD virus serotypes [9], the second was a serotype specific assay designed by IZSLER to detect isolates belonging to FMD virus O/EA-3 topotype. This virus, FMD virus O/EA-3 topotype, was circulating in the nearby region of North Africa. Primers and probes were designed on the sequences of O/EA-3 isolate in Algeria 2019. Both the reactions were conducted using the Super-Script™ III Platinum™ One-Step qRT-PCR Kit (Thermo Scientific). The amplification profile and reactions conditions was as follow: 50°C (30min), 95°C (10min), 95°C (15 sec-50 cycles), 50.3°C (1min, 50 cycles, fluorescence capture), described by Callahan et al. [9]. Primes, the probe and the profile of the serotype specific Realtime RT-PCR are shown in Table 2.

Table 2. Primers and probe for the real-time RT-PCR designed for the detection of topotype O/EA3 from Algeria.

Oligo name	Sequence 5′ to 3′
O_EA3_ALG_F	CCTCCTTCAACTACGGTG
O_EA3_ALG_R	GCCACTATCTTCTGTTT
O_EA3_ALG_P	FAM-CTGCTGGCAATTCACCCG-BHQ1

2.7. VP1 sequencing

The VP1 (636bp) amplification was performed according to Knowles et al. [10]. The first RT-PCR reaction was done by using the One-Step RT-PCR kit (Qiagen) with the following amplification profile: 30 min 50°C (RT), 15 min 95°C, 1 min 95°C (35 cycles), 1 min 55°C (35 cycles), 2 min 72°C (35 cycles), 5 min 72°C, using the primers and reaction condition shown in Table 3.

Table 3. VP1 amplification reaction using One-Step RT-PCR kit.

First amplification	For each sample (µl)
H2O	10.25
5X Buffer (2.5mM MgCl2)	5
dNTPs 10 Mm	1
Primer For (10pmoli/ul) O-1C283F (GCCCAGTACTACACACAGTACAG)	1.25
Primer Rev (10pmoli/ul) EUR-2B52R (GACATGTCCTCCTGCATCTG)	1.25
One Step MIX	1
Rnasi Inhibitor (Promega)	0.25
Total volume	20

The second reaction (emi-nested) was conducted using 5 µL of the first amplification product, using the same reverse primers of the first reaction and the primer O-1C583F (GACATGTCCTCCTGCATCTG) as forward primers. The reaction condition and amplification profile were the same as the first reaction except for RT step which was not included in the amplification profile.

The amplified product (25µL) was loaded on 2% agarose gel and, after the migration, the band corresponding to the expected molecular weight was slice off and purified using the kit NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel).

The VP1 sequencing was carried out by Sanger method using the instrument 3500XL genetic analyzer (Applied Biosystem). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 [11] that including VP1 sequences from GenBank and also from World Reference Centre at The Pirbright Institute and by ANSES.

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3. Results

3.1. Detection of serotype O of FMD virus on FTA cards

Only seven samples out of 16 eluted from impression smears of epithelium tissues on FTA cards were positive for the pan-FMD realtime RT-PCR targeted on the 3D gene (Table 4).

Table 4. Results of realtime TR-PCR with 3D gene target and Algerian FMD virus of O/EA-3 topotype.

Sample	Host	Type of sample	City	Realtime RT-PCR 3D (Ct)	Realtime RT-PCR designed on Algerian O/EA-3 2018-19 (Ct)
1	Cattle	Epithelial tissue	Misrata	30.09	31.47
2	Cattle	Epithelial tissue	Misrata	22.79	28.24
3	Cattle	Epithelial tissue	Misrata	20.23	22.13
4	Cattle	Epithelial tissue	Misrata	24.52	26.07
5	Cattle	Epithelial tissue	Misrata	21.07	28.23
6	Cattle	Epithelial tissue	Misrata	31.44	30.64
7	Cattle	Oral swab	Misrata	Undetected	Undetected
8	Cattle	Whole blood	Misrata	Undetected	Undetected
9	Cattle	Epithelial tissue	Tajoura	26.28	28.64
10	Cattle	Whole blood	Tajoura	Undetected	Undetected
11	Sheep	Oral swab	Tajoura	Undetected	Undetected
12	Sheep	Oral swab	Tajoura	Undetected	Undetected
13	Sheep	Oral swab	Tripoli	Undetected	Undetected
14	Sheep	Oral swab	Tripoli	Undetected	Undetected
15	Sheep	Oral swab	Tripoli	Undetected	Undetected
16	Sheep	Epithelial tissue	Tripoli	Undetected	Undetected

All positive samples were detected by a prototype realtime RT-PCR designed to detect FMD virus O/EA-3 topotype based on Algerian isolates O/EA-3 from the 2018-2019 FMD outbreak. Therefore, these positive seven positive samples for FMD virus serotype O and most likely to be topotype O/EA-3, that has been confirmed by VP1 gene sequencing (Figure 2).

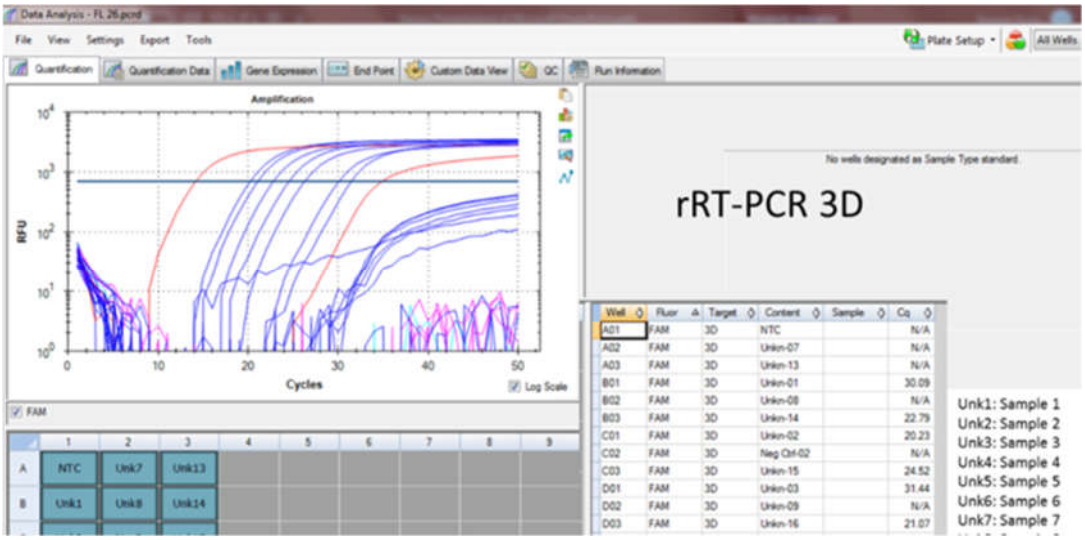

Figure 2. Amplification curves obtained with the Realtime RT-PCR based on the 3D gene.

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The result showed that seven samples out of 16 tested FTA cards (43.8%) were positive for FMD. Six out of the seven positive samples from Misrata and one positive sample was collected from Tajoura. All samples collected from Tripoli were negative for FMD. All seven positive samples on FTA cards were epithelial tissues impressions. The other nine negative FTA samples were mainly swabs and blood samples.

3.2. Identification of topotype

The detected FMD virus serotype O was confirmed to be O/EA-3 topotype by sequencing of the VP1 gene. The analysis of the VP1 sequence demonstrated that the virus belongs to topotype O/EA-3 with nucleotides identity ranging from 99.7-99.8% with the Algerian topotype O/EA-3 isolated during the FMD outbreak in Algeria in 2019 (Table 5). The phylogenetic analysis shows the high similarity between the Libyan and Algerian isolates (Figure 3).

Table 5. Most closely related sequences of Algerian FMD isolates to the Libyan isolate.

Sequence	Virus name	Host	Identity %	Serotype	Topotype
viba_41371	ALG/Medea/2019/IZSLER/19/33806/2	Cattle	99.8	O	EA-3
viba_41387	ALG/Medea/2019/IZSLER/19/33806/6	Cattle	99.8	O	EA-3
viba_41379	ALG/El Bayadh/2019/IZSLER/19/33806/4	Cattle	99.8	O	EA-3
viba_41375	ALG/Oran/2019/IZSLER/19/33806/3	Sheep	99.8	O	EA-3
viba_41383	ALG/Tissemsilt/2019/IZSLER/19/33806/5	Sheep	99.8	O	EA-3
viba_39456	ALG/1/2019	Cattle	99.7	O	EA-3
viba_39452	ALG/8/2018	Cattle	99.7	O	EA-3
viba_41367	ALG/El Bayadh/2019/IZSLER/19/33806/1	Cattle	99.7	O	EA-3

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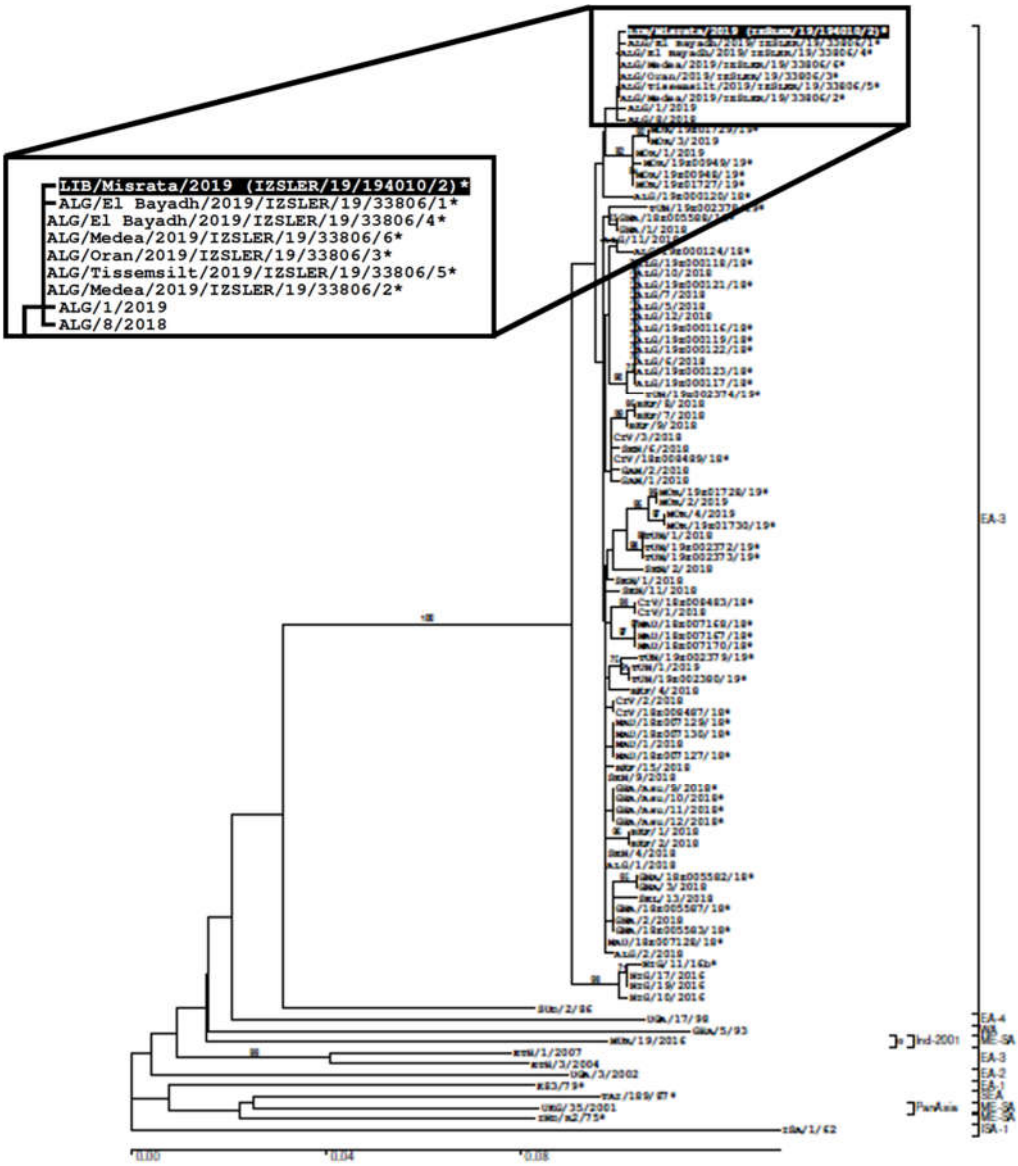

Figure 3. The phylogenetic tree analysis of the VP-1 gene showing very high nucleotide identity between the FMD virus circulating in Libya and the Algerian FMD viruses isolated in Algeria on 2018 and 2019.

4. Discussion

In the current study, we have reported and confirmed FMD outbreak in Libya by using a field tool known as FTA cards in which the nucleic material can be preserved in the card and stored in the room temperature and shipped with normal courier without the need for dry ice or cold temperature. In the laboratory, RNA of the FMD virus can be extracted from the FTA card and molecular diagnostic techniques can be done to reach the final diagnosis [12].

In the present study, the real-time RT-PCR technique was specific for the detection of FMD virus serotype O and related to O/EA3 isolate in Algeria 2019, has confirmed the presence of the genomic material in seven samples out of 16 clinical samples collected from animals showing clinical signs of FMD. These seven positive samples were mainly from epithelial tissues. Whereas, the other nine clinical samples on the FTA cards were mainly swabs and blood samples. This can be explained as the viral loads would be expected to be higher in the epithelial tissues compared to swabs and blood samples where the viral loads would be expected to be low [13].

By the analysis of VP1 sequence to those of other related viral sequences by blasting of nucleotide sequence in the gene bank, it was confirmed that the viruses detected in this study were closely related to FMD virus serotype O, with identity more than 98% Algerian FMD virus serotype O isolate (ALG/1/2018), both of which are included in topotype EA-3 that differs from the previous circulating topotype in Libya ME-SA, which was prevalent in Libya from 2013 to 2014 [14]. The observed strain is primarily found in Sub-Saharan Africa; nevertheless, topotypes of this strain were previously reported in many African countries [15]. The detection of this topotype in this study would predict that these topotypes will continue to circulate in the region and in Africa in general [5,16,17].

Libya's unique geographical location in North Africa, makes it susceptible to FMDV strains that can be spread to other countries. This was the case when the O/ME-SA/Ind-2001d sublineage was first reported in Libya in 2013, then spread to other countries in North Africa, causing many outbreaks in Tunisia related to animal movements from Libya, and then the same topotype was reported in Algeria in 2014 and then in Morocco in 2015 [18]. Furthermore, previous researchers have emphasized the potential of FMDV strains prevalent in Sub-Saharan Africa spreading to North Africa [19].

However, although serotypes O and A of FMDV are known to be endemic in Libya, with serotype O being more dominant than serotype A, the detected strain in current study during 2019 outbreak clustered independently from the previously identified viruses.

Illegal movement of livestock from sub-Saharan countries creates a warning alarm for the continuous introduction of transboundary animal diseases including FMD viruses [20]. Despite many efforts by the Libyan veterinary authority to control FMD, the shortage in proper quarantining facilities and illegal movement of animals from the surrounding countries puts Libya at high-risk of continuous disease introduction.

5. Conclusions

In countries where there are difficulties in shipping clinical samples to Reference laboratories, other alternative, cheap and applicable methods can be taken and replace the normal, expensive and complicated methods for sending clinical samples to international laboratories.

FTA cards are cheap and can preserve the genetic materials from the impression of clinical samples for long time at the room temperature and can be shipped easily and very cheap at the room temperature without the need for dry ice or cold chain.

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Conflicts of Interest: The authors declare no conflict of interest.

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