

Serological Detection of FMD Virus and Its Antibodies

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Abstract : Foot and Mouth Disease (FMD) or Aphthous fever is a fundamental global animal health trouble. FMD ranks first between the notifiable list A infectious diseases of animals. It is the most contagious transboundary animal disease (TAD) infecting cloven hoofed species of domesticated and wildlife. The most susceptible domesticated species are cattle, sheep, goats, pigs and buffalo. As the rapid accurate detection of FMD virus antigens and antibodies is an essential corner stone in the epidemiological studies, the present work aims to determine FMD virus (or antigens) in diseased cattle and FMD antibodies in vaccinated cattle using different serological tests in order to detect the most suitable one to ensure such purpose. The study include detection of FMD virus in samples from 25 naturally infected cattle and serum of vaccinated 100 animals by using serum neutralization test (SNT); Agar gel precipitation test (AGPT); Fluorescent antibody technique (FAT) and rapid slide agglutination test (RSAT) using staph protein-A. Application of AGPT revealed that 2 tongue epithelium (TE) and 2 saliva (S) samples were negative to any virus type while 10 TE and 5 S samples were positive to type O; 6 TE and 4S samples were positive to type A and 1TE and no S samples were positive to type SAT-2. One TE sample showed positive result to mixed infection with both of type O and A. Direct FAT showed that 12TE and 10S samples were positive to type O; 8TE and 7S samples were positive to type A and 3TE and 1S samples were positive to type SAT-2 without detection of mixed infection. The results of RSAT using SPA came parallel and confirmed to those of FAT. Trials of virus isolation on BHK21 cell culture revealed that FMD virus type O was detected in 5 TE samples; type A was detected in 3TE samples and type SAT-2 in 1 TE samples through the three successive viral passages while other samples were found to be negative to active virus although they were positive to AGPT, FAT and RSAT. SNT and RSAT using staph protein-A, showed that both tests revealed the same results in a parallel manner to confirm each other. Detecting FMD antibodies, it was found that the SNT consume long time (18-24 hours) while RSAT needs shortest time (2-5 minutes) showing titers similar to those obtained by SNT for the three virus types. Eventually, RSAT is the most rapid; easy; not requires special equipment or laboratory and of low cost in addition to its consideration as a field test.

Keywords – FMD, TAD, SNT, AGPT, FAT, RSAT, TE, S.

Date of Submission: 05-05-2018

Date of acceptance: 24-05-2018

I. Introduction

Foot and mouth disease (FMD) is one of the most troubles viral disease among livestock specially cloven footed domestic and wild animals including cattle, buffaloes, sheep, goats and pigs [1]. It is a highly contagious viral disease among livestock in the world in the terms of economic impact and hindering on the trade of animals on national and international level and restriction of people movement which affect the tourism sector [2]. FMD is the most important livestock disease in the world in terms of economic impact. The reason is the ability of the disease to cause losses of production, including lameness, loss of appetite, reduced milk yields, abortions, delayed conception, perinatal mortality and premature culling and also due to hindering on the trade of animals both locally and internationally, and restrictions on the movement of people which affect the tourism sector.

1.1. Historically:

FMD virus has been reported in almost of African, European and Asian countries as Kuwait, Israel, Iraq, Kingdom of Saudi Arabia, Egypt, Libya, Turkey, Japan, Korea, Bulgaria, Greece, Albania, Macedonia, Yugoslavia, Taiwan, Morocco and England [3]. It was mentioned that foot and mouth disease is one of the most dangerous diseases of cloven-hoofed animals and is a constant threat in the Middle East and other regions throughout the world despite intensive vaccination program. FMD remained-and still remains- endemic in the Middle East including the Asiatic part of Turkey (Anatolia). In recent years FMD has been reported mainly in the Balkans: in Turkish Thrace in 1995, 1996 and 2001, in Greece in 1994, 1996 and 2000 and in Bulgaria

propagated to Ireland and to continental Europe. This was the first major epidemic of FMD in Europe since preventive vaccination had been abandoned in 1991 in continental Europe [4-5]. Thomson *et al.*, (2002) indicated that 6 of the 7 types of FMD virus occur in Africa, which is a unique situation in comparison with other regions of the world [6].

1.2. The causative agent:

The causative agent of FMD foot and mouth disease is a member of genus Aphthovirus of family Picornaviridae. Picorna viral RNA genomes encode of viral polyprotein precursor, which is processed into the P1 region, containing the capsid proteins VP1, VP2, VP3 and VP4 and the P2 and P3 regions which contain the non-structural proteins [7-8]. FMDV particle is an icosahedral with 32 equal sides that are constructed from 60 copies of different virus proteins (VP1-VP4), encoding the virus RNA [9]. ICTV (2000) reported that FMD was caused by seven species (types) of foot and mouth disease in the genus Aphthovirus, family Picornaviridae. The genome is approximately 8kb in size [10].

1.3. Clinical symptoms of FMD:

Animals infected with FMD show higher fever (40-41°C); fall in milk yield, anorexia followed by the appearance of an acute painful stomatitis. There are abundant salivation, the saliva hanging in long ropy strings, and a characteristic smacking of the lips. Vesicles appear on the buccal mucosa, dental pad, tongue and rupture within 24 hours leaving a raw painful surface, which heals in about a week. Vesicles appear on the feet particularly in clefts and on the coronet. Ruptured vesicles cause acute discomfort and the animals are grossly lame with painful swelling of the coronet [11].

II. Materials and Methods

2.1. MATERIALS:

2.1.1. Animals:

Twenty five native breed calves of about 1-1.5 year; in a private farm; were found suspected to be infected with FMD. These animals were subjected to clinical examination where they found to suffer from rise in body temperature, off food, salivation with the presence of vesicles on the gums and inter digital spaces.

2.1.2. Samples:

2.1.2.1. Samples for virus (or antigen) detection:

Twenty five saliva and twenty five tongue epithelium samples were obtained from the naturally affected animals on sterile cold saline with antibiotic and transferred on ice to the lab and kept at -70°C till subjected to laboratory examination for detection of FMD virus or antigen.

2.1.2.2. Serum samples:

One hundred serum samples were obtained from vaccinated calves one month post vaccination with the locally prepared trivalent oil FMD vaccine in a private farm and subjected for estimation of their levels of FMD (O, A and SAT2) antibodies using the applied serological assays.

2.1.3. Cell culture adapted viruses:

BHK cell culture adapted FMD virus type O, A and SAT2 were supplied by VSVRI and used for monitoring of FMD antibody levels in vaccinated animals through application of target serological tests in the present study.

2.1.4-Reference anti-FMD sera virus type O, A and SAT2:

These anti-sera were kindly supplied by VSVRI and used as positive controls in the applied serological tests.

2.1.5-Cell culture:

Baby hamster kidney cell line (BHK13) was supplied by VSVRI and used in serum neutralization test and trials of FMD virus isolation of infected animals.

2.1.6-Cell culture media and solutions:

2.1.6.1- Hank's balanced salt solution (HBSS):

HBSS was prepared according to Hank and Wallace [12] and used in cell culture passage as a washing and diluent's solution for serum samples in serological tests.

2.1.6.2-Trypsine solution:

One percent solution of 1/250 trypsin (supplied by Difco laboratories, Detroit Michigan) was prepared in HBSS and used for cell culture passages as cell dispersing solution.

2.1.6.3-Sodium bicarbonate solution:

It was prepared as 4.5% solution in double distilled water and used for adjustment of the required pH values of cell culture solutions.

2.1.6.4- Antibiotic solution:

A stock solution of antibiotic solution was prepared by dissolving 1 gm of Streptomycin and 1 million IU of Penicillin-G sodium in 100 ml of HBSS. 1% of such solution was added to any of cell culture solutions on use to yield a final concentration of 100 microgram of Streptomycin and 100 IU of Penicillin G sodium/ml.

2.1.6.5-New born calf serum:

New born calf serum (virus and mycoplasma screened) was supplied by Gibco Limited Company, (Paisley, Scotland, UK, P.B. 35) and used in the ratio of 10% as a supplement for cell culture growth media while 2% of such serum was added to maintenance media.

2.1.6.6-Cell culture media:

Minimum essential media (MEM) with Hank's salts, L-glutamine and without sodium bicarbonate was supplied by Gibco (G80 Gibco Limited, P.O. Box 35 Parsley Scotland UK). It was prepared according to the manufacture directions. MEM was used with 10% new born calf serum as growth medium while it was used with 2% serum as maintenance medium for cell cultures.

2.1.7-Staph protein-A:

Staph protein-A was prepared according to Kessler [13] and Sting *et al* [14] from Cowan-1 strain of S.aureus and supplied by Prof. Dr. Saad, M. A.M (Animal Reproduction Research Institute; Alharam Giza) and used in rapid slide agglutination test (RSAT).

2.2. METHODS:

2.2.1. Collection of serum samples:

Serum samples were collected through the jugular vein puncture from vaccinated calves one month post vaccination. The sera were collected and stored at -20°C and inactivated at 56°C for 30 minutes before being used in the serological tests.

2.2.2--Serum neutralization test (SNT):

The test was performed by the microtechnique as described by Ferreira [15] in flat bottom tissue culture microtitre plates. Two fold serially diluted sera in MEM (Modified Eagle's Medium) were used. From each dilution, 50 µl per well was distributed into 4 wells and the virus was added (100 TCID₅₀ in 50 µl to each well). Neutralization was allowed to occur at 37°C for 1 hour. BHK cells were added, 150 µl per well and the inoculated cultures and control serum and virus were incubated at 37°C for 48 hours with daily microscopic examination. The SN titer of the serum was expressed as the reciprocal of the final serum dilution which neutralized and inhibited the CPE of 100 TCID₅₀ of the used virus according to Singh *et al* [16].

2.2.3-Detection of FMD and antibodies antigen using agar gel immune diffusion test (AGIDT):

The prepared sample homogenates were subjected for detection of FMD antigen or antibodies using specific reference anti-FMDV sera according to Kitching *et al* [17] where the antiserum was placed in the central well while the tested samples were placed in the peripheral wells.

2.2.4-Direct fluorescent antibody technique (FAT):

BHK cells were cultured on cover slips in Leighton's tubes then infected with the obtained FMDV and when clear CPE was observed (50-60%) some of these cultures were stained with H and E stains while others were subjected to direct FAT.

Direct FAT was carried out on infected BHK cell culture according to Florence *et al* (1992) as follow: Fixed cover slip tissue cultures (infected and non infected controls) were passed through PBS briefly and a drop of FMDV serum conjugated with FITC was placed on each cover. After half an hour incubation at 37°C in CO₂ incubator, they were rinsed 3 times in PBS (PH 7.4) then mounted in phosphate buffered glycerin (1:10) on clean slides. The preparations were examined with VEB Carl Zeiss JENA-DDR-fluorescent microscope with

HBO 202 maximum pressure mercury lamp, 12 v/50w halogen lamp, excitation filters of 50 mm diameter (to give different bands of excitation) , barrier filters of 20 mm diameter and the magnification power 500x.

2.2.5-Rapid agglutination test (RAT):

Rapid agglutination test was carried out on FMD virus type O; A and SAT2 obtained from infected BHK21 cell culture for detection of the virus antigens using specific anti-sera and on vaccinated cattle sera and reference FMD anti-sera using SPA. The test was carried out according to Montassier *et al* [18] .

2.2.6-Demonstration of FMDV cytopathic effect (CPE):

Normal and infected cell cultures prepared on cover slips were stained with hematoxylin and eosine (H&E) according to Carleton *et al* [19] to demonstrate the induced CPE of FMDV on 24 hours post cell infection.

III. Results

3.1. Clinical examination of diseased animals:

Examination of diseased calves showed generalized buccal cavity and interdigital spaces lesions in 15 calves and only mouth lesions in ten of them. They found to suffer from rise in body temperature (41-42°C), off food, salivation with the presence of vesicles on the gums and interdigital spaces. Abundant salivation was seen and the saliva hanging in long ropy strings (Fig.1) with a characteristic smacking of the lips. Vesicles appear on the buccal mucosa, dental pad and tongue and rupture leaving a raw painful surface (Fig.2). Ruptured vesicles cause acute discomfort and the animals are grossly lame with painful swelling of the coronet. These data are demonstrated in table (1).



Figure 1: FMD affected calf with saliva hanging in long ropy strings.



Figure 2: Ruptured buccal vesicles leaving a raw painful surface.

| Clinical signs | Number of affected animals | Percentage |
|---------------------------------|----------------------------|------------|
| Rise body temperature (41-42°C) | 25 | 100 |
| Salivation | 25 | 100 |
| Foot and Mouth lesions | 15 | 60 |
| Mouth lesions only | 10 | 40 |

Table 1: Detected FMD signs in naturally infected calves.

3.2. Detection of FMD virus (or antigen):

Application of AGPT; direct FAT and RSAT on the collected samples from the field naturally infected animals revealed that AGPT indicated 2 tongue epithelium (TE) and 2 saliva (S) samples were negative to any virus type while 10 TE and 9 S samples were positive to type O; 6 TE and 8 S samples were positive to type A and 7 TE and 6 S samples were positive to type SAT-2. One TE sample showed positive result to mixed infection with both of type O and A. Positive AGPT showed clear precipitation lines between the tested samples and used specific FMD virus type's antisera (Fig.3).

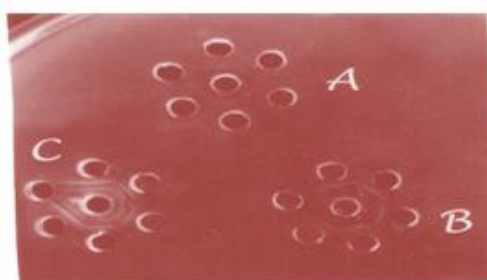


Figure 3: Positive AGPT results showing clear precipitation lines between tested samples and FMDV antisera (A=weak positive: B=moderate positive: C=strong positive).

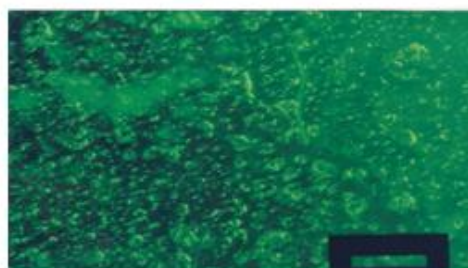


Figure 4: Positive direct FAT (100Xs) showing clear apple green reaction.

Direct FAT showed that 12 TE and 11 S samples were positive to type O; 8 TE and 9 S samples were positive to type A and 3 TE and 3 S samples were positive to type SAT-2 without detection of mixed infection. Apple green reaction was determined in case of positive FAT reactions (Fig.4).

On the other side positive results of RSAT using SPA showing clear agglutinations (Fig.5) came parallel and confirmed to those of FAT. These results are tabulated in table (2).



Figure 5: Positive RSAT using SPA showing clear agglutination.



Figure 6: BHK cell culture infected with FMD virus (either type) showing contracted cells, long cytoplasmic protrusions and detachment from the culture surface (H&E, 100Xs)

3.3. Propagation of FMD virus in BHK21 cell culture:

Samples showing positive reactions with AGPT; FAT and RSAT (12 O; 8A and 3SAT-2) were passaged three successive times in BHK21 cell cultures in order to investigate the presence of active virus. These trials revealed that type O was detected in 5 TE samples; type A was detected in 3TE samples and type SAT-2 in 1 TE samples. These findings were obtained through the three successive viral passages while other samples were found to be negative to active virus although they were positive to AGPT, FAT and RSAT the thing which may be due to the presence of viral antigen but not active virus able to propagate in cell culture.

Demonstrating the induced cytopathic effect of the obtained FMD virus samples, it was found all of them induced specific FMD virus changes in BHK21 cell culture characterized by cell contraction, elongation of cytoplasmic process followed by cell detachment from the culture surface between 18-24 hours post cell infection (Fig.6).

| Used test | Required time | (Detection of FMD virus or antigen) | | | | | |
|-----------|-----------------------|-------------------------------------|-----|-----------|-----|---------------|-----|
| | | Type O in | | Type A in | | Type SAT-2 in | |
| | | *TE | **S | *TE | **S | *TE | **S |
| AGPT | Up to 24 hours | 10 | 9 | 6 | 8 | 7 | 6 |
| FAT | Not less than 2 hours | 12 | 11 | 8 | 9 | 3 | 3 |
| RSAT | 2-5 minutes | 12 | 11 | 8 | 9 | 3 | 3 |

Table 2: Detection of FMD virus or antigens in examined field samples.

*TE= tong epithelium **S= saliva

| *Antibody titer | Using SNT | Using RSAT |
|---------------------|----------------------------|----------------------------------|
| | Number of positive samples | Number of positive samples |
| 4 | 5 | ↑ The Same Results ↓ |
| 8 | 6 | |
| 16 | 14 | |
| 32 | 26 | |
| 64 | 25 | |
| 128 | 24 | |
| Total sample number | 100 | 100 |
| Required time | 18-24 hours | 2-5 minutes |
| Requirements | Special laboratory | Field test |

Table 3: FMD type O antibody titer in vaccinated calf sera one month post vaccination.

| *Antibody titer | Using SNT | Using RSAT |
|---------------------|----------------------------|----------------------------------|
| | Number of positive samples | Number of positive samples |
| 4 | 0 | ↑ The Same Results ↓ |
| 8 | 32 | |
| 16 | 31 | |
| 32 | 9 | |
| 64 | 13 | |
| 128 | 15 | |
| Total sample number | 100 | 100 |
| Required time | 18-24 hours | 2-5 minutes |
| Requirements | Special laboratory | Field test |

Table 4: FMD type A antibody titers in vaccinated calf sera one month post vaccination.

| *Antibody titer | Using SNT | Using RSAT |
|---------------------|----------------------------|----------------------------------|
| | Number of positive samples | Number of positive samples |
| 4 | 2 | ↑ The Same Results ↓ |
| 8 | 16 | |
| 16 | 22 | |
| 32 | 25 | |
| 64 | 15 | |
| 128 | 20 | |
| Total sample number | 100 | 100 |
| Required time | 18-24 hours | 2-5 minutes |
| Requirements | Special laboratory | Field test |

Table 5: FMD type SAT2 antibody titers in vaccinated calf sera one month post vaccination.
FMD type A antibody titers in vaccinated calf sera one month post vaccination.

3.5. Determination of FMD antibody levels in vaccinated animals:

Serum neutralization test (SNT) and rapid slide agglutination test (RSAT) showed the same results confirming each other for each of FMD antibodies type O; A and SAT2. Regarding FMD type O antibodies it was found that the antibody titer was determined as 4 (5 samples); 8 (6 samples); 16 (14 samples); 32 (26 samples); 64 (25 samples) and 128 (24 samples) as shown in table (3).

FMD type A antibody titers were 8 (32 samples); 16 (31 samples); 32 (9 samples); 64 (13 samples) and 128 (15 samples) as tabulated in table (4).

FMD type SAT2 antibodies showed the titers of 4 (2 samples); 8 (16 samples); 16 (22 samples); 32 (25 samples); 64 (15 samples) and 128 (20 samples) as demonstrated in table (5).

Although the 2 tests indicate the same results; RSAT is the faster one requiring 2-5 minutes while SNT requires 18-24 hours to obtain a definite result.

IV. Conclusion

Depending on the present obtained results it could be concluded that:

- 1-Foot and Mouth Disease is still threaten cattle population in Egypt with the three virus types (O; A and SAT2).
- 2-Animal vaccination is the corner stone for disease control.
- 3-The local trivalent FMD oil vaccine is safe and potent able to protect cattle against natural infection where it induces good levels of specific immunity in vaccinated animals.
- 4-Detection of FMD virus or antigens and antibodies could be carried out through application of different tests as AGIDT; FAT; RSAT; SNT and propagation in BHK21 cell line.
- 5-RSAT is the most rapid; easy; not requires special equipment or laboratory and of low cost in addition to its consideration as a field test.

Acknowledgment

This research project would not have been possible without the support of many people. I would like to express my sincere thanks to my guide, Prof. Dr. Mokhtar Mohamed Ali El-Tarabili, Prof. of Virology; Faculty of Veterinary Medicine, Suez Canal University. I deeply appreciate the vast knowledge that I earned from him.

I was truly fortunate to conduct this study under the supervision of Prof. Dr. Mohamed Hassan Khodeir, Consultant Chief of Researches; Veterinary Serum and Vaccine Research Institute, I want to express my sincere gratitude for his academic supervision, guidance, and for all his efforts and technical support.

I pay my deep sense of gratitude to Prof. Dr. Hesham Mohamed Shafik, Prof. of microbiology to encourage me to the highest peak. His prompt inspirations, timely suggestions with kindness, enthusiasm and dynamism have enabled me to complete my thesis.

I would also like to thank all staff members of Veterinary Serum and Vaccine Research Institute for their support through the journey to accomplish this work, and special thanks to Dr. Ahmed Al-behwar for helping me.

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Eman, A. Mohamed "Serological Detection of Fmd Virus and Its Antibodies ." *IOSR Journal of Agriculture and Veterinary Science (IOSR-JAVS)* 11.5 (2018): 10-16.