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# Molecular epidemiology of Foot and Mouth Disease Virus during 2014 with References to Biochemical Changes in Egyptian Buffaloes

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## ABSTRACT

***In April 2014 foot-and-mouth disease virus (FMDV) affected water buffaloes (Bubalus bubalis) aged from 3-5 years in Qalyubia, Egypt. The aim of the present study was to diagnose FMDV molecularly and biochemically. Blood samples were collected from buffaloes suffering from characteristic clinical signs of FMDV infection as fever, profuse ruby threads salivation, ulcer on muzzle, vesicles on foot and lameness. Blood samples, tongue epithelium and vesicular fluid were evaluated by real time RT-qPCR for the diagnosis of FMDV using different probes and primers of universal (3D) gene and VP1 gene for serotypes A, Iran O, Asia and SAT2.***

***The positive sample confirmed by one step reverse transcription polymerase chain reaction (RT-PCR). This resulted in the identification of a SAT2 serotype was the causative agent and the amplified RNA virus resulted in 716bp. Serum samples of positive PCR infected animals compared with apparently healthy control group was used to determine the concentration of aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP), albumin, total protein, calcium (Ca), iron (Fe) and inorganic phosphorus (Ph). A level of nitric oxide (NO) and malondialdehyde (MDA) were calorimetrically measured in serum as markers for oxidant status. There was a significant increase (P<0.05) in AST, ALT, ALP, Ph, NO and MDA and a significant decrease (P<0.05) in albumin, total protein, Ca and Fe in serum of clinically affected animals. It was concluded that FMDV serotype SAT2 circulate in Egypt and associated with biological alteration and disturbed oxidative status.***
***Key words: Molecular, FMDV, Buffaloes, Biochemical and Oxidative.***

## INTRODUCTION

Foot and mouth disease virus (FMDV) is a highly contagious disease of all cloven foot domestic and wild animals. It caused by the family *Picornaviridae* of genus *Aphthovirus* (**Yang et al., 1999 and Mezencio et al., 1999**). There are seven serotypes of FMD virus A, C, O, SAT1, SAT2, SAT3, and Asia 1. Infection or vaccination with one serotype does not confer immunity against the others. Thus, the virus causing the outbreak must be isolated and characterized for an appropriate vaccine selection **(Biswal et al., 2014).** FMDV is endemic in most countries in Asia, like India, Iran and Pakistan as well as in sub-Saharan Africa and Egypt (**Salem et al., 20**12).

The disease is characterized by fever blisters or vesicles erosions and ulcers in the mucosa of mouth, tongue lips, gums, pharynx and palate. Vesicles may also found on the coronary band between claws and on the teats, lameness is evident in animals with foot lesions, some strains of the virus cause necrosis of heart muscles and many result in death before lesions develop in the more common and visible locations such as the mouth or foot (**Rodostits, 2007 and Lubroth, 2002**).

FMD is enzootic in Egypt, due to the O and A serotypes (**Knowles et al., 2007 and FAO, 2012**). In February 2012 SAT 2 was the primary cause of a FMD outbreak struck Egypt and led to 82362 suspected cases, of which 19655 died (**FAO, 2012**).

The molecular epidemiology of FMDV has progressed from a study of European outbreaks, often closely associated with re-introductions of vaccine strains to global comparisons of many genetic lineages and the detailed study of a single pandemic strain (**Knowles and Samuel, 2003**). Lineage-Specific Reverse Transcription-Polymerase Chain Reaction Amplification Assays (RT-PCR) useful for screening large numbers of FMDV isolates in order to confirm the presence of a single genetic lineage or to distinguish two or more variants active in a single geographical location (**Knowles and Davies, 2000**). RT-PCR procedures have been evaluated at the world reference laboratory (WRL), Pirbright, UK for the routine diagnosis of FMD virus using universal primers for all seven serotypes and serotype-specific primers **(Reid et al., 2000)**. Currently, molecular methods to detect FMDV are mostly used to confirm or rule out FMD in suspected cases. The only method routinely employed is RT-PCR, usually in the form of a real-time assay (**Moniwa et al., 2007**).

Viral infection activities the immune system, it causes release of reactive oxygen species (ROS) and reactive nitrogen species (RNS) with the potency of inducing oxidative stress (**Mousa and Galal, 2013**). Detection of free radicals damage and the body's defenses it has become increasingly macrophages to synthesize large quantities of nitric oxide (NO) that plays an important role as defense mechanism (**Rockett et al., 2007**), it has cytotoxic effects on these activators when synthesized in large quantities (**Kandmir et al., 2011**). ROS and RNS are capable of degrading portion and nucleic acid. In addition it can attack the polyunsaturated fatty acids of membrane lipids causing lipid per oxidation lead to disorganization of cell structure and function (**Halliwell et al., 1992**). Lipid per oxidation is well established mechanism of cell as injury and is used as an indicator of oxidative stress in cells and tissues (**Mousa and Galal, 2013**). The most abundant lipid peroxide by product is malondialdehyde (MDA) **(Heidarpour et al., 201**3). It used as indicative markers for oxidative damage (**Kandemir et al., 2011**). The body minimized the cellular effects of ROS by production of antioxidants which depleted with increasing of ROS production **(Zalba et al., 2006**).

This study was designed for the diagnosis of FMD serotypes by real time RT-qPCR and confirmed by RT- PCR on symptomatic blood samples to investigate the genetic characteristic, biological alteration and oxidative stress parameters associated with FMD disease virus in apparently infected buffaloes.

## MATERIALS AND METHODS

### Animals

The study was carried out on 100 dairy buffaloes aged 3-5 years in different private farms in Qalyubia Governorate were suffering from characteristic clinical signs suggested to be due to FMDV infection during April 2014 as mentioned in **(Table, 1**). All animals subjected to clinical examination according to **(Radostits et al., 2007).**

### Samples

Two types of blood samples were collected from jugular veins of symptomatic infected buffaloes. The first type of blood samples were collected in sterile tubes with EDTA for amplification by PCR as well as tongue epithelium and vesicular fluid. The other type of blood samples were collected in tubes and left few minutes to obtain serum for serologic evidence of biochemical and oxidative profiles. The collected samples were coded and preserved at -20 °C till used.

### Genetic characterization

### Extraction of viral RNA

Genomic RNA was extracted from blood samples, epithelium and vesicular fluid using EZ1 Virus Mini Kit by EZ1 Advanced Automatic Extractor (Qiagen–Germany) as instructed by the manufacturer's protocols.

### Primers and probes selection for genotyping of FMDV

Oligoprimers and probes for universal (Callahan 3D) gene for common FMDV were designed according to **(Callahan et al., 2002)** for detection of all seven serotypes of FMDV and the most variable capsid protein includes a major immunogenic site of the virus (VP1) gene serotypes A, Iran O, Asia and SAT2 **(Ferris et al., 2009)** have been used to genotype the topotypes of FMDV by one step real time RT-qPCR were listed in **Table (2).**

**Quantitative Real time (RT- qPCR)**

One step real time RT-qPCR was performed for the diagnosis of FMDV topotypes. Thermal profile at 55°C for 5min as RT-PCR, enzyme inactivation at 95°C for 10min followed by 45 cycling at 95°C for 15sec for denaturation and at 60°C for min for annealing /extension step where data collection by fluorescence was measured **(Table, 3).** Positive and negative controls were including for each serotype in micro Amp optical 96-well reaction plate (Applied Biosystem). Positive sample was subsequently amplified by conventional RT-PCR.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Assays**

Oligonucleotide two pairs of primers of FMDV SAT2 were designed according to **Ried et al., (2000)** was illustrated in **Table (4).** Complementary DNA (cDNA) was prepared from the extracted viral RNA. A one-step RT-PCR assay was performed using QIAGEN® One Step RT-PCR Kit in Biometra T1 Thermocycler according to the instruction of the manufacturer. Reverse transcription (RT) were performed at 50°C for 30min, Taq inactivation at 95°C for 15min for one cycle, followed by 35 cycling including denaturation at 95°C for 60sec, annealing at 50°C for 60sec and extension at 72°C for 2min and then final extension at 72°C for 2min. Negative controls (PCR-grade $H_2O$ without template) was incorporated with each set of test samples and subjected to PCR assays to avoid the number of false-positives resulting using a safety cabinet. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel stained with 0.5µg/mL ethidium bromide and observation under short-wavelength UV light. The positive result is indicative at 716bp.

**Biochemical Examination**

Biochemical changes were determined in positive RT-PCR blood samples and compared by apparently healthy buffaloes (10 in No.) was used as control group. Colorimetric determination, aspartate amino transferase (AST), alanine amino transferase (ALT) and alkaline phosphatase by using kits provided from (Diamond Diagnostics, Egypt). Serum albumin and total protein (Gamma Trade Company, Egypt). Calcium (Ca), iron (Fe) and inorganic phosphorus (Ph) (Bio-Diagnostic, Giza, Egypt). The concentrations of oxidant makers NO (**Montgomery and Dymock, 1961**) and MDA (**Satoh, 1987**) were measured and normal range in buffaloes' serum sample was used as reference, **Table (5).**

**Statistical analysis:**

Data were computed and statistically analyzed using student "t" test and Chi, Square Analyses (**Snedecor and Cochran, 1980**).

## RESULTS

Infected buffaloes with FMD virus disease showed clear clinical signs including vesicles and erosion in tongue, ruby salivation, ulcers in hoof and ulcers in buccal cavity (**Figure, 1**). The most observed clinical signs were fever, ulcer on muzzle and vesicles on foot and 70% showed more than one clinical signs (**Table, 1**).

Detection of FMDV using real time RT-qPCR targeting to universal (Callahan 3D) gene and VP1 gene serotypes (O, A, Asia and SAT2) were positive to primer and probe for both common (3D) FMDV and serotype SAT2, but failed to amplify other serotype VP1 specific primers. The real-time RT- qPCR cycle showed an increase in the fluorescence signal is detected initially as threshold cycle ($C_T$) which is proportional to the amount of the specific PCR product. The positive control gave $C_T$ value at 12 and the positive samples gave $C_T$ values at 12, 22, 24, 26,

27, 30 and 32, while the negative control gave no curve (**Figure, 2**). Thus, testing the positive samples by SAT2 conventional RT-PCR yielded positive results and amplified expected size at 716bp (**Figure, 3**). Neither negative control nor the negative reactions showed bands. No non-specific band or laboratory contaminations were detected.

Biochemical profile and oxidative status associated with positive RT-PCR FMDV in diseased buffaloes compared with apparently healthy control group were calorimetrically determined **(Table, 5)**. Level of AST, ALT, ALP, Ph, NO and MDA were significant ($P<0.05$) increased, while level of albumin, total protein, Ca and Fe were significant ($P<0.05$) decreased in serum of infected animals.

## DISCUSSION

FMDV is highly contagious due to the ability of the causative agent to gain entry and initiate infection via a variety of sites, the small infective dose, the short incubation period, and the release of FMDV before the onset of clinical signs. In addition, the massive quantities of virus excreted from infected animals, its ability to spread large distances due to airborne dispersal and the survivability of the virus in the environment contribute to its contagiousness (**Sanson et al., 2011).**

The current study was carried out during April 2014 as 100 buffaloes aged 3-5years showed characteristic signs of FMD infection and 10 apparently healthy buffaloes used as control group. The clinical examination revealed that animals suffered from high fever, depression, dullness, anorexia, ruby threads salivation, panting, lameness, and vesicular eruptions on buccal mucosa and inter digital space in addition to appearance of vesicles on mucous membranes of the mouth including tongue, dental pad, gum, lips and teats and drop in milk yield. Similar clinical observations were recorded in cloven foot animals in Egypt **(El-Ashmawy et al., 2013, Zaher and Ahmed, 2008 and Mousa and Galal, 2013 )** and African countries, East Africa **(Sutmoller et al., 2003),** Uganda **(Ayebazibwe et al., 2010),** Tanzania **(Magoma et al., 2000, Catley et al., 2004)** and Ethiopia **(Rufael et al., 2008).**

The fever attributed to replication of the FMD virus in central nervous system of affected animals leading to disturbance in heat regulatory centrals **(Bhattacharya et al., 2005).** The emaciation and loss of condition may be attributed to the loss of body weight resulting from difficulty in eating and walking to food (**Meyer and Knudsen, 2001).** The affected buffaloes showed also congested mucosa that could be attributed to the pyrexia as the rectal temperature reached 41.6C in affected animals (**Radostits et al., 2007).**

Seven serotypes of FMDV are known; serotypes O and A are widely distributed, and the Southern African Territories (SAT) serotypes (1, 2, and 3) usually are restricted to Africa. Serotype Asia 1 has never circulated within Africa; serotype C has not been identified anywhere since 2005 **(Sangula et al., 2011).** Recently FMDV serotypes SAT 1 and SAT 2 viruses were successfully isolated from clinically normal African buffalo (*Syncerus caffer*) in Kenya (**Wekesa et al., 2015).**

The present work showed that the real-time RT- qPCR was conducted for primary diagnosis of FMD virus and their serotypes on field samples for topotype of FMDV. Positive samples showing curve of high cycle threshold values in serotype SAT2 and negative samples for FMDV serotypes A, O, and Asia1 gave no curve. Our results are in harmony with **(Abd El Wahed et al., 2013).** The results of real time RT- qPCR assays indicated that the serotype responsible for the endemic is

SAT2 and confirmed by 716 bp amplified product using RT-PCR. This result agreement with the newly introduced SAT-2 serotype, which has been emerged during February to April 2012 in Upper Egypt **(Salem et al., 2012)** Delta Governorates **(Ahmed et al., 2012 and Valdazo Gonzales et al., 2012),** Gharbia (**Elhaig and Elsheery 2014**) and Alexandria (**El-Shehawy et al., 2014)**. Moreover, the failure in detection of serotypes A and O not prevent their implications in field cases (**Salem et al., 2012)**. On the other hand our findings are supported **by (Kandeil et al., 2013)** who reported that serotypes O and A were under control by vaccination in Egypt.

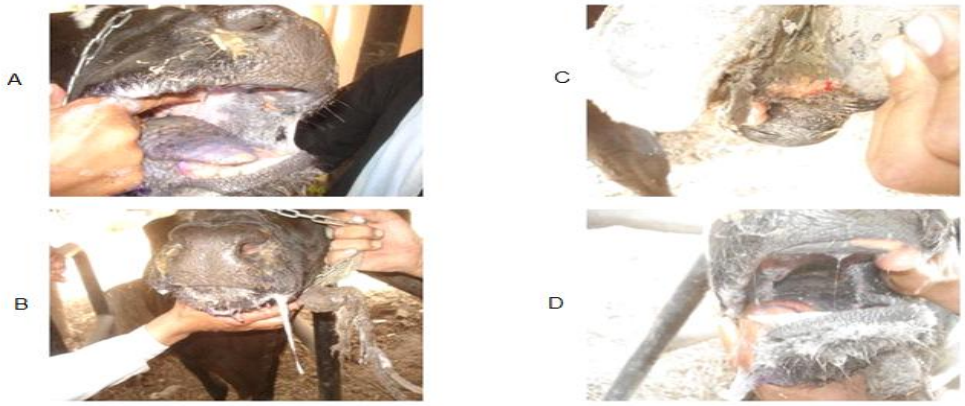
Regarding enzymatic and biochemical parameters in FMDV diseased buffaloes, showed significant (P value < 0.05) decrease in total protein, albumin and calcium level. A result came in parallel with that mentioned by **(Gokce et al., 2004, Mohapatra et al., 2005 and Krupakaran et al., 2009**).

Hypoprotenemia and hypoalbumin could be a result from sever anorexia and off food due to oral lesions as mentioned by **(Kaneko et al., 1997).** Protein requirement and protein catabolism increase in the presence of infection or any lesion in body as recorded by **(Meyer and Harvey, 1998).** Hypocalcaemia could be attributed to sever anorexia and hypoproteinemia in affected cattle resulting in decrease protein bounded calcium as recorded by **(Gokce et al., 2004).**

The affected buffaloes declared significant increases (P <0.05) in the Ph level similar to that reported by **(Mohapatra et al., 2005).** Hyperphosphatemia may be attributed to the increased salivation with the resultant dehydration and decreased renal blood flow **(Gruenberg et al., 2005).** Moreover, the increased Ph level could also be response to hypocalcaemia because of the interaction between the Ca and Ph homeostasis in ruminants **(Breves and Schroder, 1999).** Therefore, the hyperphosphataemia could be response to the change in relative proportions of ionized Ca and Ph in blood **(Thilsing et al., 2007).**

The level of Fe was significantly reduced in diseased group when compared to control. This could be attributed to anorexia **(Kaneko et al., 1997).** The significant (P<0.05) increase of enzymes such as ALT, AST and ALP suggest that FMDV may be associated with damage to the liver, muscles or other organ **(Ghanem and Abdel-Hamid, 2010).** However, **Gokce et al., (2004)** reported no significant changes in ALT, AST and ALP in cattle affected with acute FMD infection.MDA and NO are significantly increase (P <0.05) and considered the most bio- product of lipid peroxidation and markers of oxidative stress. These results agree with **(Lee et al., 2004)**. Therefore, the significant increase in MDA level in diseased group suggested that FMDV might induce oxidant injury **(Gokce et al., 2004).** ROS and nitrogen metabolites play a complex role in many infectious diseases; such metabolites influence the growth of viruses by inhibiting replication of virus **(Sen and Packer, 1996 and Wischral et al., 2001**). Oxidative stress resulted from the faster production of reactive forms of oxygen than its safely scavenging by the antioxidant mechanism and it has negative effect on animal health and productivity as well as it has been implicated as major initiator of tissue damage **(Bernabucchi et al., 2002 and Ahmed et al., 2005**). Macrophages, neutrophils and other phagocytic calls considered as the potent cells of immune response against viral and microbial infections. Those cells generated large amount of ROS and RNS that considered as the main cause of lipid peroxidation lipid peroxidation is used as an indicator of oxidative stress in tissues. Significant high level of NO production suggests that FMDV induce production of NO. This result agreed with over production of ROS leading to occurrence of oxidative stress, oxidative stress has been implicated as major indicator of tissue damage**.** Moreover FMDV is associated with disturbed

oxidative status and sub functional ovarian activity in buffalo-cows **(Zaher and Ahmed, 2008).** The negative correlation between albumin level MDA and NO may be due to its antioxidant properties of albumin **(Castillo et al., 2005).** This antioxidant function of albumin is attributed to multiple legend radical trapping properties of it **(Oettl and Stauber, 2007 and Mousa and Galal, 2013).**




Clinical signs of foot and mouth disease in buffaloes
a-vesicales and errosion in tongue
b-| Ruby salivation
c-Ulcers in hoofs
d-Ulcers in buccal cavity

**Figure 1. Clinical signs associated with FMD virus disease in buffaloes.**

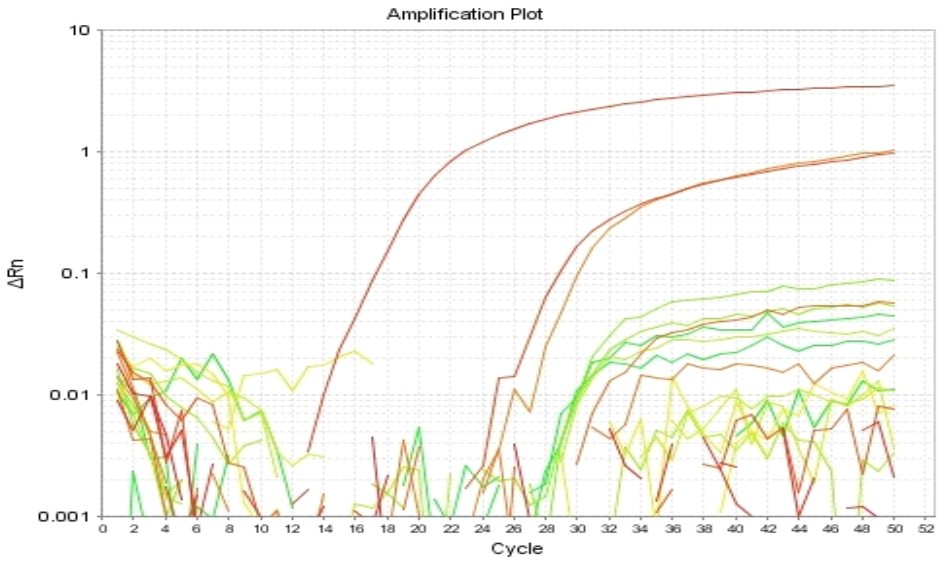



**Figure 2. Amplification of the conserved region, for FMDV and topotype SAT2 using Real time RT- qPCR (Applied Biosystems). Linear regression analysis of cycle threshold ($C_T$) values of amplification curves representing positive samples examined for the detection of FMD virus with $C_T$ values at 12, 22, 24, 26, 27, 30 and 32, while negative one gave no curve. The positive control gave $C_T$ value at 12.**

**Table 1. Percentage of the most clinical signs associated with FMD virus disease.**

| number<br><br>Clinical Signs | No. of clinically affected buffalo | Percentage |
|---|---|---|
| **Fever** | 53 | 53% |
| **Off food** | 37 | 37% |
| **Lameness** | 52 | 52% |
| **Salivation** | 28 | 28% |
| **Ulcers on muzzle** | 58 | 58% |
| **Vesicles on foot** | 58 | 58% |
| **Vesicles on teat** | 30 | 30% |
| **More than one clinical signs** | 70 | 70% |

**Table 2. Primers and probes for genotyping of FMDV by Real Time RT- qPCR.**

| FMDV | Gene | Direction | Oligonucleotid sequence (5′ – 3′) |
|---|---|---|---|
| **Universal** | Callahan 3D | Forward | ACT GGG TTT TAC AAA CCT GTG A |
| | | Reverse | GCG AGT CCT GCC ACG GA |
| | | Probe | TCC TTT GCA CGC CGT GGG AC |
| **A Iran** | VP1 | Forward | ACG ACC ATC CAC GAG CTY |
| | | Reverse | RCA GAG GCC TGG GAC AGT |
| | | Probe | CGT GCG CAT GAA ACG TGC CG |
| **O** | VP1 | Forward | CCG AGA CAG CGT TGG ATA |
| | | Reverse | CCA TAC TTG CAG TTC CCG |
| | | Probe | CCG ACT TGC ACT GCC TTA CAC GGC |
| **Asia** | VP1 | Forward | GCA GTW AAG GCY GAG ASC |
| | | Reverse | GCA RAG GCC TAG GGC AGT |
| | | Probe | AGC TGT TGA TCC GCA TGA AAC GYG CG |
| **Sat 2** | VP1 | Forward | TGA AGA GGG CTG AGC TGT |
| | | Reverse | CTC AAC GTC TCC TGC CAG |
| | | Probe | ACA GAT TCG ACG CGC CCA TCG |

**Table 3. Thermal cycling protocol for detection of FMDV by Real Time RT- qPCR.**

| Operation | Temp. | Time | Cycle |
|---|---|---|---|
| RT | 55$^{o}$C | 5min. | 1 |
| Enzyme inactivation | 95$^{o}$C | 10min | 1 |
| Denaturation | 95$^{o}$C | 15sec | 45 |
| Hybridization, extension and data collection | 60$^{o}$C | 1min | |

RT: Reverse transcriptase.

**Table 4. Primer design for RT- PCR.**

| FMDV | Primer design | Sequence (5'-3') | Genome location | |
|---|---|---|---|---|
| | | | Gene | Position |
| SAT2 | SAT-1D209F | CCACATACTACTTTTGTGACCTGGA | VP1 | 209-234 |
| | SAT-2B208R | ACAGCGGCCATGCACGACAG | 2B | 208-227 |

**F:** Forward. **R:** Reverse.

**Table 5. Biochemical and oxidative status associated with FMD virus disease. T-test Values are expressed as mean and mean of standard error, values with different superscriptions (a and b) in rows differ significantly (P<0.05).**

| Groups<br>Parameters | Infected Group (n=100) | Control Group (n =10) | Normal Range (Reference) |
|---|---|---|---|
| **AST** u / l | **20.70± 0.9644$^{b}$** | **6.615 ± 1.473$^{a}$** | **6.9 - 35** |
| **ALT** u / l | **163.0 ± 10.30$^{b}$** | **104.0± 2.12$^{a}$** | **60 - 125** |
| **ALP** u / l | **122.9± 17.90$^{b}$** | **58.57 ± 1.53$^{a}$** | **18 - 153** |
| **Albumin** g / L | **2. 13± 0.235$^{b}$** | **2.72 ± 0.801$^{a}$** | **2.5 - 3.8** |
| **Total protein** mg / L | **3.382 ± 0.352$^{b}$** | **5.450± 0.3350.$^{a}$** | **6.7 - 7.5** |
| **Ca** mg / dl | **7.571± 0.3244$^{b}$** | **10.23± 0.1877$^{a}$** | **8 - 11** |
| **Iron** ug / dl | **59.29± 0.5055$^{b}$** | **89.30 ±19.36$^{a}$** | **57 - 162** |
| **Ph** mg / dl | **9.110 ± 0.308$^{b}$** | **7.946 ± 0.3365$^{a}$** | **5.6 - 8** |
| **NO** nmol / L | **35.3 ± 0.10$^{b}$** | **23.82± 7.63$^{a}$** | **18 - 21** |
| **MDA** nmol / ml | **9.450 ± 0.864$^{b}$** | **4.194 ± 0.941$^{a}$** | **1.23 - 2.14** |

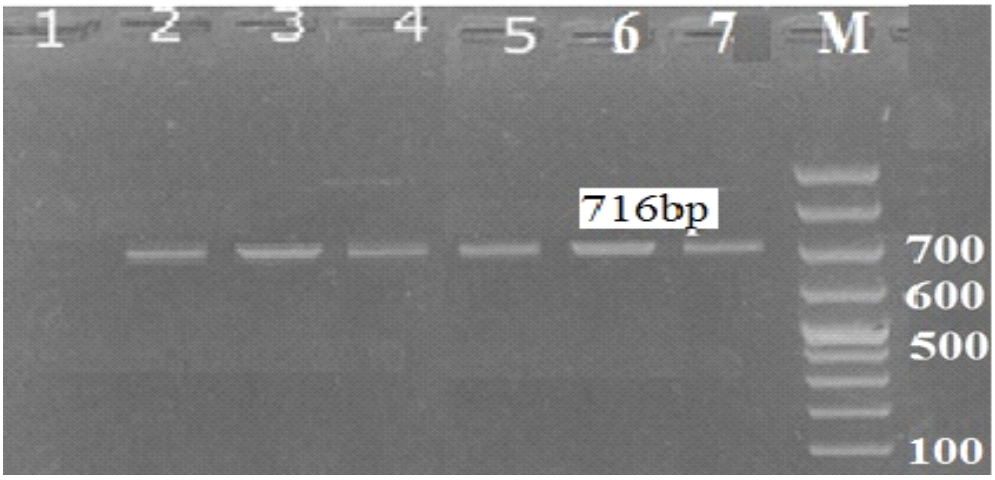



**Figure 3. RT-PCR amplification of the 716bp products of RNA extracted from blood, tongue epithelial and vesicular fluid samples of buffalo for detection of FMDV- SAT2. M: 100bp molecular weight markers. Lane (1): Negative control. Lane (2): Positive control. Lanes (3 to 7): Positive samples.**

## CONCLUSION

Our study provides evidence of the circulation of SAT2-type FMD virus among buffalo populations. The most diagnostic methods in FMDV outbreak are clinical signs supplemented by PCR to determine strain. Strong oxidative stress associated to FMDV, so antioxidant and immune stimulants drugs recommended during treatment of viral disease.

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