Epidemiological and Clinicopathological Studies of Sheep Naturally Infected with Foot and Mouth Disease Virus (SAT2) in Egypt

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ABSTRACT

Key words: FMDV, SAT2, Sheep, Egypt, Cardiac Biomarker.

This study was done to evaluate the epidemiological pattern and cardiac markers of foot and mouth disease virus (FMDV, SAT2) in naturally infected sheep, in Dakahlia Governorate, Egypt. The specific antigen ELISA test confirmed positive FMDV, SAT2 in sheep with oral lesions (14.7%), lameness (22.9%) and in dead sheep (3.97%). Death was highest (p<0.001) in the age group 1-2 months. Serum samples were collected and categorized based on the survival outcomes (survival or death). Serum from clinically healthy group of sheep was collected and used as control. The assessment of myocardial injury was based on the determination of cardiac troponin I (cTnI), creatine kinase (CK), lactate dehydrogenase (LDH) and aspartate transaminase (AST). Cardiac troponin I (cTnI) and CK levels of the death group were significantly (p < 0.0001) higher than the survival group. The results were supported by histopathological examination that revealed myocarditis and myocardial necrosis. In conclusion, the cause of deaths in sheep with foot and mouth disease (SAT2) may be due to the myocardial injury and cTnI analysis may be used as an early diagnostic biomarker.

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1. INTRODUCTION:
Foot and mouth disease (FMD) is a highly contagious acute vesicular disease of cloven-hoofed animals. The etiological agent is Foot and mouth disease virus (FMDV) (Belsham, 1993). The virus belongs to the Aphthovirus genus of the family Picornaviridae with seven clinically indistinguishable serotypes: O, A, Asia 1, SAT 1, SAT 2, SAT 3 and C (Dukpa et al., 2011). Foot and mouth disease impacts negatively the livestock industry in countries where it is endemic, or in the event of re-introduction in already-free regions (Correa Melo et al., 2002). The disease continues to pose threat to the livestock sector in the world (Sharma et al., 2015)

The epidemiology of FMD in North Africa is complicated by the co-circulation of endemic FMDV, as well as sporadic incursions of exotic viral strains from the Middle East and Sub-Saharan Africa (Ahmed et al., 2012). And also due to animal movement, diverse farm practices and large number of susceptible livestock in the country. (Sharma et al., 2015) The disease was recorded in Egypt since 1950, whereby serotypes SAT2, O and A were identified. Serotype A and SAT2 were the main causes of outbreaks during 1953, 1958 and 1960 and since then, serotype O was the only FMDV strain circulated in livestock in Egypt until 2006 (Ghoneim et al., 2010). In 2006, serotype A outbreaks were reported in cattle and buffalo (Knowles et al., 2007; OIE, 2006). In September 2007 and January 2008, serotype O outbreaks were reported in Alexandria and El Buhayrah governorates (FAO, 2008). In 2012, SAT2 outbreak was reported in Egypt in cattle and buffaloes (Ahmed et al., 2012).

Sheep have an important role in the epidemiology of FMD and are the main cause for introduction of the disease to a free country (Kitching and Hughes, 2002). Naturally acquired FMD infection often takes a milder form in sheep than in cattle or pigs. Viraemia may be present for up to 3 days before the appearance of vesicular lesions (Alexandersen et al., 2003). Deaths in lambs start to occur 2–3 days after the appearance of clinical signs in ewes. Heart failure or starvation are reported as the main cause of deaths. Post-mortem lesions included myocarditis, septicemia, abomasitis and enteritis (Littlejohn, 1970). Myocardial injury is predominant
Troponin is a complex protein that regulates actin myosin interaction in the thin filaments of striated muscle fibers and is essential for calcium regulatory system of muscle contraction. It is composed of three subunits (C, T, I) (Bodor et al., 1995; Ware, 2011). It has been suggested that cTnI might be the choice specific biomarker of myocardial damage even in a minimal focal injury (Antman et al., 2000). Cardiac injury could be indicated by elevation of troponin levels but the mechanism has not been described (Babuin and Jaffe, 2005). Rising of cardiac troponin was recorded in human with myocardial infarction (Antman et al., 2000; Gupta and de Lemos, 2007), cattle with traumatic reticuloenteritis and with different cardiac injury (Gunes et al., 2008; Serra et al., 2010; Varga et al., 2009), sheep after induced anterior coronary artery legation (Leonardi et al., 2008), calves and lambs with FMD (Karapinar et al., 2010; Mohri and Movassaghi, 2013) and goats kids with myocardial injury (Tharwat et al., 2013).

Serum CK, AST and LDH were increased with myocardial pathologies (Bertinchant et al., 2000; Bleuel et al., 1995; Ooi et al., 2000). Although, creatine kinase myocardial band (CK-MB) was described as specific myocardial injury biomarker (Wallace et al., 2004), it peaked and returned to the normal level within 24 hours (h) in case of myocardial infarction (Rajappa and Sharma, 2005).

Serum cTnI had 6 h half-life after peaking at 4-6 h following isoproterenol-induced cardiac toxicity in rat (O’Brien et al., 2006). It was elevated at 12 h and peaked at 24 h in monensin-induced myocardial toxicity in cows. While CK was increased at 72 hours and reached the maximal concentration at 80 hours. There was a relationship between cTnI and total heart score, but no correlation was found with CK (Varga et al., 2009). Therefore, cTnI had higher sensitivity and specificity than CK for the detection of myocardial injury (Korff et al., 2006; Varga, 2008).

The objective of this study was to evaluate the epidemiology and clinical pathology of FMDV SAT2 in sheep in Dakahlia Governorate of Egypt with focus on cardiac injury markers.

2. MATERIALS AND METHODS:

2.1 Animals and sampling protocol:
Sheep (840) from different locations (3) A, B, and C. Different flocks (10) and of different ages groups (Table 1) were selected for the study. Age of the animals was based on responses from flock men or owners. The sheep had been reared together with cattle herds in Dakahlia governorate, Egypt during the last FMD outbreak in 2012. The sheep had not been vaccinated against FMD a year ago, before the study begun. The clinical examination of the animals was based on (fever, oral lesions, heart examination and lameness) and record of mortalities were done according to (Kelly, 1990; Lovatt, 2010).

2.2. Samples:

2.2.1. Jugular blood samples were collected from all affected sheep at 1st day post appearance of the clinical signs. Blood samples from five apparently healthy sheep served as control group based on the clinical examination. Serum samples were carefully separated and stored at -20°C until used for serum neutralization test to rule out the previous infection or vaccination by SAT2 virus. From 1st age group (1-2 months) and 2nd age group (3-6 months) group, serum samples were categorized into two main groups: survival and death groups, then 10 and 5 serum samples (1/3 number of each group) were randomly selected from each respectively, in addition to the control one for determination of cTnI, AST, CK and LDH levels.

2.2.2. Epithelial tissue specimens (gum, dental pad, hard palate, cheeks and tongue from dead sheep) and vesicular fluid before rupture of the vesicle were collected by sterile syringe in sterile tubes with phosphate buffer solution with 50% glycerol. In addition, vesicular swabs from recent ruptured vesicles were collected. The samples were kept at -80 °C till used for detection and typing of the FMDV.

2.2.3. Tissue specimens (heart, tongue, gums) from freshly dead lambs were fixed in 10% formalin for histopathological examination.

2.3. Antigen Detection and Serotyping of FMDV
Antigen detection and serotyping of FMDV (O, A, SAT1, SAT2) were performed using FMDV antigen detection and serotyping of FMDV (O, A, SAT1, SAT2) according to the manufacturer procedures (IstitutoZooprofilatticoSperimentaledellaLombardia e dell’Emilia Romagna [IZSLER], Biotech Laboratory Brescia, Italy; (Ferris et al., 2011). The tests for detection and serotyping of the FMDV in
sheep were carried out at Central Laboratory for Evaluation of Veterinary Biologics, Abbasia, Egypt.

2.4. Serum Neutralization Test

Serum neutralization test was done according to (Ferreira, 1976). Simply, the used FMD strains were SAT2/EGY/2012, O/PAN ASIA2 and A/IRAN-05 obtained kindly from Central Laboratory for Evaluation of Veterinary Biologics, it was performed in flat bottomed tissue culture microtitre plate 96 wells. Serum samples were serially diluted (1:4) in modified Eagle Medium (MEM), 50 µL from each dilution was distributed into the wells and 50 µL of 100 TCID50 FMDV were added to each well, then the plates put on shaker for 10 minutes. Then it was incubated at 37 °C for one hour, 150 µL of BHK21 cell suspension were added to each well, in each plate cell control and virus control, then the plate was sealed with pressure sensitive adhesive cellulose tape. Plates were incubated at 37C for 48hrs and reading by inverted microscope, the serum neutralization titer was expressed as log 10 of the reciprocal dilution which protected 50% of the cells as calculated by Karber method (1931).

2.5. Serum Biochemical Parameters

Serum cTnI was analyzed by the immune enzymometric assay (for human) using anti-cTnI mouse monoclonal antibodies (ST AIA-PACK cTnI 3rd-Gen, Cat. No. 0025215) by Tosoh Bioscience, Inc., on TOSOH AIA 360 automated immunoassay analyzer, TOSOH HI-TEC INC. The assay sensitivity threshold for cTnI was 0.02 ng mL-1. Spectrophotometric analysis was carried for serum AST, CK and LDH on A15 Biosystems (Cod No 1-002) analyzer using kits of Biosystems S.A. (Spain). All procedures followed manufacturers’ instructions. The analysis was carried out at the Department of Biochemistry, Faculty of Medicine, Mansoura University.

2.6. Histopathological Examination

Tissue specimens were processed by conventional paraffin embedding techniques and stained by hematoxylin and eosine for routine histopathological examination (Harris, 1998).

2.7. Statistical Analysis

The statistical analysis system, SAS 9.2 for windows, (SAS Institute Inc., U.S.A) was used for analysis of serum neutralization test, cTnI, AST, CK and LDH. One-way analysis of variance (ANOVA) and Duncan’s multiple-range tests were performed. Data were presented as means, ± standard deviations (SD), and the significance difference was defined as P ≤0.05.

3. RESULTS

3.1 Clinical Examination:
The clinical presentation of sheep suffering from FMD is shown in table (2) and Fig. (1, 2 and 3) The signs included fever, oral lesions in the oral cavity on the cheeks, hard palate, dental pad, gums and tongue, lameness which was the first sign to be observed in the flocks. Mortalities were recorded specially in young lambs at age of 1 -2 months (11 dead lambs) and in age group 3-6 months (4 dead lambs).

3.2 Serum Neutralization Test

The lowest level of serum neutralization antibodies were recorded in age group 1-2 months with higher (P≤0.05) levels recorded in the other age groups (Table 3).

3.3 Antigen Detection and Serotyping of FMDV

SAT2) was demonstrated in sheep naturally infected with FMD in epithelial tissues and vesicular fluid of the examined sheep in all groups and negative for types O, A and SAT1.

3.4 Estimation of Serum Biochemical Parameters:
The concentration of cTnI, AST and CK levels were significantly (P≤0.05) higher in the death group than control and survival groups Table 4). However, the increase of LDH was significantly (P≤0.05) higher in death group than control group.

3.3. Histopathological Examination

Macroscopical postmortem examination of dead lambs revealed presence of necrotic lesions and ulceration in the oral cavity and the heart showed myocardial necrosis (tiger heart) (Fig.10). Histopathological examination of the myocardium revealed lymphocytic infiltration, edema and necrosis. The tongue showed retained fluid in the epithelium (vesicle), with necrosis and desquamation of the superficial layer, with intense lymphocytic infiltration in lamina propria (Fig. 4, 5,6,7,8 and 9).

4. DISCUSSION

The present study demonstrated severe oral lesions, lameness and mortalities in lambs during the FMD (SAT2) outbreak in cattle in Egypt 2012 Table (2) and Fig. (1,2,3). The severe clinical signs observed
may have been due to exposure of the sheep to SAT2 virus for the first time.

Table (1): sampling protocol of clinical suspected FMD sheep.

<table>
<thead>
<tr>
<th>Area (n=3)</th>
<th>Flocks (n=10)</th>
<th>Total number (n=840)</th>
<th>Number of clinical suspected animals (n=353)</th>
<th>percentage of clinical suspected animals (42.02)</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>A1</td>
<td>90</td>
<td>34</td>
<td>37.78</td>
<td>1-2 months (70)</td>
</tr>
<tr>
<td>A</td>
<td>A2</td>
<td>89</td>
<td>31</td>
<td>34.83</td>
<td>8</td>
</tr>
<tr>
<td>A</td>
<td>A3</td>
<td>101</td>
<td>35</td>
<td>34.65</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>280</td>
<td>100</td>
<td>35.71</td>
<td>20</td>
</tr>
<tr>
<td>B</td>
<td>B1</td>
<td>70</td>
<td>33</td>
<td>47.14</td>
<td>7</td>
</tr>
<tr>
<td>B</td>
<td>B2</td>
<td>75</td>
<td>31</td>
<td>41.33</td>
<td>5</td>
</tr>
<tr>
<td>B</td>
<td>B3</td>
<td>90</td>
<td>34</td>
<td>37.78</td>
<td>8</td>
</tr>
<tr>
<td>B</td>
<td>B4</td>
<td>65</td>
<td>33</td>
<td>50.77</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>300</td>
<td>131</td>
<td>43.67</td>
<td>25</td>
</tr>
<tr>
<td>C</td>
<td>C1</td>
<td>87</td>
<td>39</td>
<td>44.83</td>
<td>8</td>
</tr>
<tr>
<td>C</td>
<td>C2</td>
<td>91</td>
<td>42</td>
<td>46.15</td>
<td>9</td>
</tr>
<tr>
<td>C</td>
<td>C3</td>
<td>82</td>
<td>41</td>
<td>50.00</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>260</td>
<td>122</td>
<td>46.92</td>
<td>25</td>
</tr>
</tbody>
</table>

Table (2): Clinical observations in Sheep Naturally Infected with FMDV.

<table>
<thead>
<tr>
<th>Age Groups</th>
<th>Total Examined Sheep</th>
<th>Oral Lesions</th>
<th>Lameness</th>
<th>Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+Ve %</td>
<td>+Ve %</td>
<td>+Ve %</td>
</tr>
<tr>
<td>1-2 months</td>
<td>70</td>
<td>11</td>
<td>15.7%</td>
<td></td>
</tr>
<tr>
<td>3-6 months</td>
<td>50</td>
<td>9</td>
<td>18.0%</td>
<td>10</td>
</tr>
<tr>
<td>7-12 months</td>
<td>76</td>
<td>18</td>
<td>23.7%</td>
<td>12</td>
</tr>
<tr>
<td>&gt;12 months</td>
<td>157</td>
<td>14</td>
<td>8.9%</td>
<td>59</td>
</tr>
<tr>
<td>Total</td>
<td>353</td>
<td>52</td>
<td>14.7%</td>
<td>81</td>
</tr>
</tbody>
</table>

Oral lesions were present in all age groups of sheep (14.73 %) and the vesicular lesions did not develop in approximately 25% of infected sheep, similar to observations by other authors (Gibson 1984; Hughes et al., 2002). Lameness was restricted to 22.9 % of the sheep and was restricted to older sheep and not all infected sheep showed a combination of vesicular and lameness despite lameness being the first noticeable FMD clinical sign (Kitching and Hughes, 2002). Mortalities were higher in lambs 1-2 months old with no previous clinical signs having been observed and this may have been due to myocardial involvement and heart failure (Kitching and Hughes, 2002; Pay, 1988).

There was a relationship between occurrence of clinical signs and age, the highest mortality rate occurred in age group 1-2 months, no lameness was observed and had the lowest oral lesions. The highest number of oral lesions was found in age group 7-12 months and no mortalities were observed. The age group more than 12 months had highest (37.6%) occurrence of lameness, with no mortalities.

The detection of antibodies against FMDV Table (3) serotypes O and A in age group 1-2 months may be due to maternal antibodies while detection of antibodies in other age groups may be due to previous infection. The absence of antibodies to SAT2 virus indicates no previous exposure to SAT2 or vaccination by a vaccine containing SAT2, so, we can specify the present clinical signs of FMDV (SAT2) in sheep and its pathological lesions for SAT2 virus, not to other strains A, or O which already circulate in the country and at the same time included in the locally produced bivalent vaccine for cattle in Egypt at that time.
The SAT2 detection based on ELISA confirmed infection of sheep with the same circulating strain in cattle (Kandeil et al., 2013). This further supports the role of sheep in harboring and transmission of FMDV between cattle and sheep (Ahmed et al., 2012; Kitching and Hughes, 2002). Our results clearly demonstrated that the serum cTnI levels increase significantly (p < 0.0001) in dead lambs with FMD in comparison with the control and survival groups. Similarly, an increase in serum cTnI levels 2 days after heart infarction has been reported in sheep (Chachques et al., 2004). In addition, the results are similar with other studies that demonstrated elevation of serum cTnI level (cTnI; 2.40-30.00 ng mL−1) in dead lamb due to FMD (Mohri and Movassaghi, 2013) but the ranges greatly differ. The results of specific assays show differences to various troponin forms because different assays measure different fragments of cTnI(Panteghini et al., 2001). In human, the lack of standardization and differences in the different assays led to major concerns regarding cTnI determinations (Zaninotto et al., 2004). Occasional false-negative results may appear cTnI(Eriksson et al., 2005). The cTnI assay could be used for diagnosis and prognosis of myocarditis in lambs in combination with ECG (Mohri and Movassaghi, 2013). High cardiac tissue reactivity (as a function of homology of tissue troponin) with human cTnI in dogs, calves, horses, sheep, and pigs has been reported (O’Brien et al., 1997). Furthermore, cross reactivity of the commercial anti-human antibody anti-cTnI with the ovine polypeptide in an experimental model has been observed (Leonardi et al., 2008). Therefore caution must be taken when using different assays in different animal species (Apple et al., 2008) and type of troponin (Jaffe et al., 2006). Few studies estimated cTnI levels in animals using commercial human kits and based on different assays such as white muscle disease in lambs (using human cTn-I immunochromatographic strip assay) (Gunes et al., 2010) and in a calf (Karapinar et al., 2010) and also in lambs with FMD (Karapinar et al., 2012). There is suspicion for cTnI diagnostic value which may depend on the disease or conditions (Ataollahi et al., 2013).

The results revealed significantly higher levels of CK in the death group as compared to other groups. The American College of Cardiology and the European Society of Cardiology recognizes myocardial necrosis with elevation of different proteins in the circulation due to the myocytes damaged: cardiac troponins, creatine kinase and
lactate dehydrogenase. In case of myocardial damage, CK should be relatively higher (at least twice the upper reference limit than those for cardiac troponin (Antman et al., 2000).

However, total CK should be accompanied with more sensitive biomarker like cardiac troponin or CK-MB for accurate myocardial infarction diagnosis (Jaffe et al., 2006). The elevation of AST levels in the death group as compared to other groups may be attributed to muscle damage and stress condition (Kaneko et al., 1997). The LDH levels were higher in death group as compared to control group only and since there are no accepted grading system of infarct size, AST and LDH should not be used for myocardial infarcts (Antman et al., 2000). Myocardial pathologies were considered a reason for elevation of serum CK, AST and LDH (Bertinchant et al., 2000; Bleuel et al.,
The significant elevation of most biochemical parameters (cTnI, AST, CK) in the death group than survival one may ensure that the reason was not due to dehydration.

Our results of the histopathological examination Fig.(4,5,6,7,8,9) supported the biochemical analysis and were in harmony with other (Harris, 1998) who reported retained fluid in epithelium (vesicle) with necrosis and desquamation of superficial layer and neutrophilic infiltration, in addition to loss of superficial epithelial layer (erosion), with intense lymphocytic infiltration in lamina propria of the freshly dead lambs tongue. Also, multi-focal areas of lymphocytic myocarditis (Ryan et al., 2008).

5. CONCLUSION
In summary, the outbreak of FMDV, SAT2 in both cattle and sheep with severe infection and clinical signs informs the veterinary authorities on the role of sheep in harboring and transmission of the FMD virus between herds as a main source of infection in the country. While sheep are no targeted in vaccination programs, the results indicate that for effective control of FMD in Egypt, vaccination of sheep should be carried out in parallel with cattle vaccination. Death of infected sheep, mostly the young ones, may have been due to myocardial injury. The cTnI commercial human kits assay may be helpful in the early diagnosis of myocardial pathologies or and this may support euthanasia in some cases.

6. ACKNOWLEDGEMENT
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