

Evaluation of cardiac enzymes and acute phase response as biomarkers for rapid diagnosis of myocarditis in calves with FMD

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Abstract

Troponin-I, homocysteine, Creatine kinase-myocardial band, lactate dehydrogenase and acute phase response had been evaluated in calves with myocarditis due to FMD. The study was conducted on 52 local breed calves 1-6 months old and from both sexes, their dams have no history for vaccination against FMD and show classical foot and mouth disease signs. Ten clinically healthy calves of the same ages were considered as controls. Suspected calves neither show oral blisters, rope salivation, nor foot lesions. Diseased calves showed signs of dullness, in activity, panting with mouth breathing, unable to suck, recumbency, However, five of diseased calves were died within 24-72 hours and on macroscopic examinations of autopsied animals, necrotic myocarditis with pale foci with a zone of hyperemia which were present on the papillary and ventricular cardiac muscles, moreover, on histopathological examinations there were severe inflammatory cells infiltration in the interstitial of myocardial fibers with obvious area of coagulation of myocardial fibers and marked area of hyalinization, furthermore, severe mononuclear cells infiltration, mainly lymphocytes, with few neutrophils closed to necrotic myocardial fibers were also detected. Diagnosis of FMD virus was confirmed by using commercially NSP ELISA kits for foot and mouth. A significant increase ($p<0.05$) was encountered in body temperature, respiratory and heart rates in diseased animals than in controls, Furthermore, abnormal cardiac sounds (organic murmurs) were indicated on auscultation of the heart. Results of hematological parameters shown a significant increase indicated in ESR values of diseased calves than in controls, moreover, total leukocyte count was increased significantly with significant lymphocytosis. Furthermore, the results were also showed significant increase in values of serum cardiac troponin, homocysteine, creatine kinase-myocardial band, lactate dehydrogenase, haptoglobin and fibrinogen in seropositive calves for FMD compared with controls. It can be concluded that determination of cardiac biomarkers and acute phase response concentration in calves with myocarditis can considered as a guide to quantify early heart damage.

Keywords: Myocarditis, FMD, Cardiac enzymes, Calves

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تقييم خمائر القلب واستجابة الطور الحاد كمؤشرات حيوية للتشخيص السريع لتهاب عضلة القلب الناتج عن مرض الحمى القلاعية في العجول

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الخلاصة

قيمت خمائر التروبونين والهيموسستين مع خمائر القلب الأخرى فضلا عن استجابة الطور الحاد في عجول تعاني من التهاب عضلة القلب الناتج عن الإصابة بمرض الحمى القلاعية، أذ شملت هذه الدراسة فحص ٥٢ من العجول المحلية بأعمار ١-٦ شهر ومن كلا

الجنسين ولم تحصن أمهاتها ضد مرض الحمى القلاعية وأظهرت العلامات السريرية الخاصة بالمرض فضلا عن ذلك فإن العجول المريضة لم تظهر العلامات السريرية الخاصة بمرض الحمى القلاعية ولكنها عانت من البلادة، قلة النشاط، اللهاث مع تنفس بطني مع عدم القدرة على الرضاعة، الرقود مع نفوق بعضها خلال ٢٤-٧٢ ساعة. فحصت عشرة من العجول المحلية وبنفس الأعمار إذ عدت كمجموعة سيطرة. تبين من الفحص العياني للعجول النافقة وجود التهاب عضلة القلب النخري مع بؤر شاحبة محاطة بمنطقة محتقنه في العضلات الأذينية والبطينية للقلب كما لوحظ في مقاطع الفحص النسجي وجود ارتشاح لخلايا التهابية في المنطقة الخلالية مع تواجد مناطق واضحة التخثر ذات تنكس زجاجي للألياف العضلية القلبية فضلا عن تواجد خلايا لمفية وعدلات بالقرب من المناطق المتخرقة. اعتمد تشخيص المرض مختبرياً على فحص الأليزا إذ تبين أن جميع العينات المفحوصة كانت موجبة للفحص كما سجلت نتائج الدراسة وجود ارتفاع معنوي في معدلات درجات حرارة الجسم، ترداد التنفس وضربات القلب في العجول المريضة بالمقارنة مع السليمة. فضلا عن سماع أصوات قلبية غير طبيعية (أصوات عضوية) عند تسمع القلب. تبين من الفحوصات الدموية حدوث زيادة معنوية في معدلات تثقل كريات الدم الحمر في العجول المريضة بالمقارنة مع السليمة فضلا عن الزيادة المعنوية في العدد الكلي لخلايا الدم البيض بسبب زيادة الخلايا اللمفية، كما تبين من نتائج الدراسة أيضاً حدوث ارتفاع معنوي في خمائر التروبونين والهيموسستين والكرياتين كينيز واللاكتات ديهيدروجينيز والهابتوكلوبين ومنتشئ الليفين في العجول المريضة بالمقارنة مع السليمة. يستنتج من هذه الدراسة أن تحديد وتقييم المؤشرات الحيوية القلبية واستجابة الطور الحاد للعجول المصابة بالتهاب عضلة القلب الناتج عن مرض الحمى القلاعية قد يعد دليلاً لتحديد حجم الضرر في القلب في وقت مبكر.

Introduction

Although Cardiovascular diseases are more important in human, however, it could be encountered in veterinary medicine as well (1). Biomarkers are biological tools used to identify high-risk individuals, For the purpose of speed and accuracy of disease diagnosis, nevertheless, it might also could determine treatment plans and prognosis, moreover the biomarkers were providing a powerful and dynamic approaches to understand the spectrum of some diseases with applications in observational and analytic epidemiology and haphazard clinical trials (2).

Foot and mouth disease is a viral disease affecting all cloven footed animals and characterized by oral vesicular stomatitis and associated with *Aphthovirus* (classified in the family *Piconaviridae*) which have seven major serotypes, including O, A, C, southern african territories SAT1, SAT 2, SAT 3 and Asia 1, however, there are a number of serologically and immunologically distinguished subtypes with different degrees of virulence (3).

High mortalities due to FMD in young animals are associated with acute myocarditis, hyaline degeneration, necrosis of muscle fibers and an intense infiltration mainly of lymphocytes, moreover, in calves, myocarditis is always considered as a fatal form of the disease that occurs mostly without development of the specific and characteristic blister lesions which can be observed by adult cattle (4). Furthermore, a higher occurrence hazardable myocarditis induced by FMD is currently known in young animals than in adults (5,6).

Diagnosis of myocardial disease in cattle still difficult and might be extremist and based upon clinical examinations of the diseased animal, since auscultation of the chest and heart, and the incidence of sudden death in the field might apocalypse for the clinical diagnosis of FMD,

thereby the use of some biomarkers for assessment of myocardial damage is advised as well (6). Biomarkers such as troponins, especially cardiac troponin-I (cTnI), Homocysteine (Hcy) and creatine kinase myocardial band (CK-MB), can be used to confirm the diagnosis because it has nearly absolute myocardial tissue specificity and higher sensitivity, furthermore, they might express and reflected the reality of the myocardial effects and pathogenesis (7).

FMD was diagnosed in most parts of Iraq, as it seems to be an endemic disease (8). Since the first record of a specific FMD, A serotype in Iraq was serotype A in 1952, Other serotypes have been reported since then, serotypes O, SAT-1 and Asia1 were recorded in 1957, 1962, and 1975 respectively, however, a lot of control programs including annual vaccination had been applied (9). The main purpose of the present study is to evaluate the biochemical biomarkers and acute phase response of local breed calves affected with myocarditis due to FMD in Basrah Governorate, Iraq, with further assessment of clinical and histopathological features of diseased and autopsied animals.

Materials and methods

Study design and animals

The study was conducted on 52 local breed calves 1-6 months old and from both sexes, their dams have no history of vaccination against FMD and showed classical foot and mouth disease signs. Suspected calves neither showed oral blisters, rope salivation, nor foot lesions. However, all examined calves had heart problems (murmur sounds, irregular heart rhythm, tachycardia and tachypnea) on auscultation. However, other diseased animals show signs of, dullness, inactivity, panting with mouth breathing, unable to suck and recumbency. Ten clinically healthy local

breed calves were considered as controls. All animals underwent a comprehensive clinical examination. The study was started from March 2017 to May 2018 in all parts of Basrah governorate.

Collection of samples

Ten milliliters of blood 10 ml were withdrawn from each calf via puncture of the jugular vein and from these (2.5) milliliter of blood mixed with EDTA used to determine hemoglobin concentration (Hb), Total erythrocyte count (TRBc), packed cell volume (PCV), and Total leukocyte count (TLC), using (Hematology analyzer from Genex, USA). Furthermore, the absolute differential leukocyte count was done using Giemsa stain blood smear method according to (10). Moreover, the erythrocyte sedimentation rate (ESR) was also estimated according to (11).

Diagnosis of FMD virus

Serum was examined for confirming the diagnosis of FMD immunoglobulins using commercially NSP ELISA kits for foot and mouth disease surveillance, according to manufacture instructions (Qucking biotech Co. Led. China).

Evaluation of acute phase response and Biochemical analysis

Serum was evaluated for Homocysteine (Hcy) (Homocysteine commercial kit from Axis® Homocystein EIA-UK), Troponin (cTnI), Troponin I, ELISA kits, Diagnostic Automation / Cortez Dianostics, INC, USA), Creatine kinase-myocardial band (CpK-MB) and Lactate dehydrogenase (LDH) (spectrophotometer using commercial kits, Roche Diagnostics, Indianapolis, GMBH, Germany). Moreover, estimation of acute phase response, including evaluation of Haptoglobin (Haptoglobin Elisa method) (Biotechnology co-china) and Fibrinogen time (using plasma) (Biolabo / France) were also done according to manufacture instructions of the producers.

Gross post mortem examination and Histopathology

Examined animals which have recently died and /or slaughtered were subjected to post mortem and laboratory histopathological evaluations after owner's approval. Heart lesions were detected and investigated, moreover, the tissue samples were collected from the interventricular, the right and left atria, and ventricular parts of the hearts, fixed at 10% neutral buffered formalin solution for 72 hours, trimmed to suitable sizes, washed, then dehydrated and cleared in xylol, then it was embedded in paraffin wax, then sectioned at 4-5 μ thickness, stained with hematoxylin and eosin, and examined under a light microscope (12).

Statistical analysis

The statistical analysis was applied according to (13) and expressed as mean \pm SD. The significance of variations between diseased and healthy calves was evaluated through SPSS student t-test.

Results

The diseased calves show signs of dullness, inactivity, panting with mouth breathing, unable to suck, recumbency, however, some of diseased calves have died within 24-72 hours after onset of signs, and by macroscopic examinations of autopsied animals, necrotic myocarditis with pale foci with a zone of hyperemia were indicated on the papillary and ventricular cardiac muscles (Figure 1). Moreover, by histopathological examinations there were severe inflammatory cell infiltration in the interstitial of myocardial fibers with obvious areas of coagulation of myocardial fibers (coagulative necrosis of myocardial fibers) and marked area of hyalinization, in addition, severe mononuclear cell infiltration mainly lymphocytes, with few neutrophils closed to necrotic myocardial fibers were also detected (Figure 2 and 3).

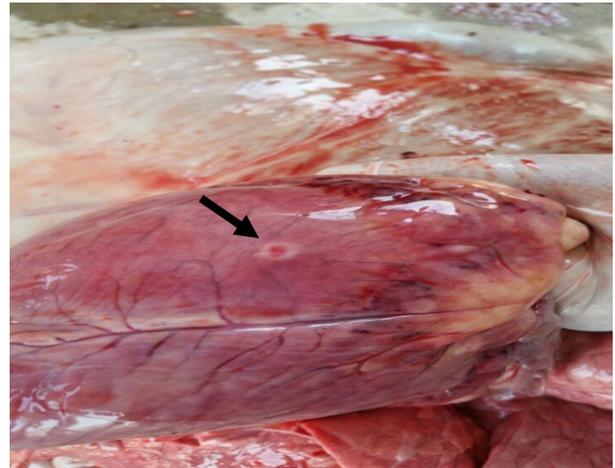


Figure 1: Gross section in the heart of FMD infected calf showing necrotic myocarditis with pale foci associated with a zone of hyperemia on the papillary and ventricular myocardium (black arrow).

The diagnosis of FMD virus was confirmed by using commercially NSP ELISA kits for foot and mouth disease and the results showed that all suspected calves were found seropositive (Figure 4).

Significant increase was encountered in body temperature, respiratory and heart rates in diseased animals than in controls, furthermore, abnormal cardiac sounds

(organic murmurs) were indicated on auscultation of the heart (Table 1).

Results of hematological parameters showed a significant increase ($p<0.05$) indicated in ESR values and total leukocyte count of diseased calves than in controls with significant lymphocytosis (Table. 2).

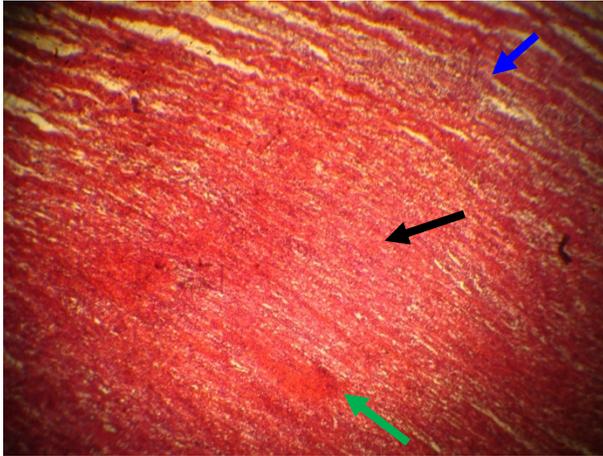


Figure 2: Histological section of the heart of FMD infected calf showing severe inflammatory cell infiltration in the interstitial of myocardial fibers (blue arrow), with obvious areas of coagulation of myocardial fibers (black arrow) and marked area of hyalinization (green arrow). H&E stain, 100x.

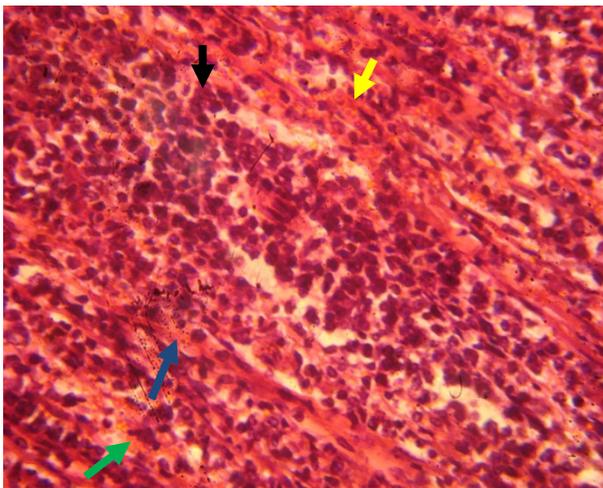


Figure 3: Histological section of the heart of FMD infected calf, showed severe mononuclear cell infiltration mainly lymphocytes (black arrow), with a few neutrophils closed to necrotic myocardial fibers (blue arrow), also noticed area of hyaline degeneration of some myocardial fibers (green arrow), in addition there are obvious areas of coagulation of myocardial fibers (yellow arrow). H&E stain, 400x.

Moreover, the results were also showed significant increases ($p<0.05$) in values of serum cardiac troponin, homocysteine, creatine kinase-myocardial band, and Lactate dehydrogenase in diseased calves with FMD than in controls (Table 3).

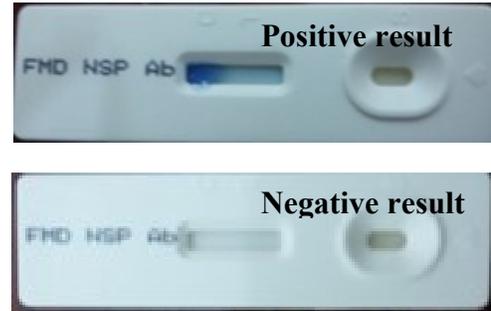


Figure 4: NSP ELISA kits (positive result for foot and mouth disease).

Table 1: Body temperature, respiratory and heart rates of diseased calves with FMD and controls

Parameters	mean \pm SD	
	Controls n=10	Diseased calves n=52
Body temperature $^{\circ}$ C	38.6 \pm 0.82	40.2 \pm 0.66**
Respiratory rate/ min	24.3 \pm 4.67	40.7 \pm 8.34**
Heart rate/ min	113 \pm 3.28	144.5 \pm 7.12**

Table 2: Hematological parameters of diseased calves with FMD and controls

Parameters	mean \pm SD	
	Controls n=10	Diseased calves n=52
RBC $\times 10^6$	7.82 \pm 1.53	7.68 \pm 1.43
Hb g/dl	12.82 \pm 2.73	12.88 \pm 3.12
PCV %	33.82 \pm 3.17	34.22 \pm 5.11
ESR mm/24h	12.34 \pm 2.65	42.67 \pm 7.91**
TLC $\times 10^3$	11.34 \pm 2.56	14.57 \pm 2.46**
Neutrophils	5233 \pm 321.16	5428 \pm 563.23
Lymphocytes	5102 \pm 462.22	8334 \pm 211.56**
Monocytes	545 \pm 362	533 \pm 267
Eosinophils	392 \pm 25	390 \pm 76
Basophiles	81 \pm 66	78 \pm 55

The data concerning acute phase response indicated a significant increase ($p<0.05$) in both Haptoglobin level and Fibrinogen time in diseased calves compared with controls (Table 4).

Table 3: Serum cardiac troponin I (cTnI), homocysteine (Hcy) and enzyme activities of diseased calves with FMD and controls

Parameters	mean ± SD	
	Controls n=10	Diseased calves n=52
cTnI ng/ml	0.21±0.06	22.91 ± 1.7**
Hcy µmol/L	5.23±0.43	14.13±2.51**
CpK-MB U/l	77± 43	255 ± 23 **
LDH U/l	118 ±31	296 ±31 **

Table 4: Haptoglobin and fibrinogen level in diseased calves with FMD and controls

Parameters	mean ± SD	
	Controls n=10	Diseased calves n=52
Haptoglobin g/dl	0.016. ± 0.006	0.056± 0.017**
Fibrinogen / sec	19.85 ± 7.67	38.91± 8.32**

Discussion

Foot and mouth disease are an important viral disease affected cloven footed animals lead to high mortalities and high economic losses, especially when the control measure was absent (3). There are different viral, bacterial and parasitic causes of infectious myocarditis in domestic animals specially in bovine, moreover, hereditary factors, vitamin E and selenium deficiency, toxicity with monensin and other causes might also play a big role as noninfectious etiology for myocardial injuries, on the other hand, high mortalities do not occur in diseased animals with FMD unless myocardial injuries which was resulting in acute death, particularly in younger age animals (4,14).

In the present study diseased animals showed different clinical manifestations which described by others (5,7). However, symptoms and severity of FMD might vary between breeds of animal and sometimes within breed, probably because of the serotype and strain of the virus, genetic factors, or the immune status of the animals (8,9,15).

The dams of the calves in the current study have no previous history of vaccination against FMD and were showed the classical signs of the disease, therefore, the primarily or a presumptive diagnosis of myocarditis due to the disease was made although the diseased calves had no vesicular and erosive lesions of FMD, whereby both myocarditis and FMD were confirmed firstly based on serological diagnosis of the non-structural proteins (NSP) ELISA test, hereby the use of (NSP) allows detection of antibodies against FMD virus in infected non vaccinated animals, as the use of (NSP) allows detection of antibodies

that are mostly only produced during infection where virus has replicated, furthermore, the possibility that some animals will show no clinical signs of disease but will have had replication of virus, such animals may carry the FMD virus and possibly represent a threat to other animals (16,17). Moreover, confirmation of FMD myocarditis could also base on the macroscopic and microscopic features of died or slaughtered animals subjected to post mortem examination, since results of this study were indicated necrotic myocarditis with pale foci and a zone of hyperemia on the papillary and ventricular cardiac muscles, furthermore, though, histopathological examinations there were severe inflammatory cells infiltration in the interstitial of myocardial fibers, accordingly a coagulative necrosis of myocardial fibers and marked area of hyalinization were also confirm the disease, same results were also described and confirmed by (4,18).

There are different chemical materials considered as a biomarker for evaluation of myocarditis especially in human (19), However, in bovine medicine, there might be no specific biochemical biomarkers for the diagnosis of myocardial injuries till now as they thought, due to lack of tissue specificity and sensitivity (20). Nevertheless, the best cardiac biomarker for myocardial damage is cardiac troponins (I or T) because they have nearly absolute myocardial tissue specificity and higher sensitivity than others, as cardiac troponins are proteins that control the calcium-mediated interaction between actin and myosin, allowing contraction at the sarcomere level, Moreover, troponin I is biomarkers responsible for inhibition of the actomyosin ATPase (21). Serum cardiac troponins may be determined during myocardial damage as the earliest appearing biochemical markers (22,23). The unique aspect of cTnI being 100% tissue specific for the heart makes it an excellent marker to serve as a biochemical and immunohistochemical tool for detecting myocardial injury, the continuing cellular release and clearance of cTnI account for its excellent diagnostic sensitivity (24). Moreover, analysis of cTn I and T, cTnT, are considered the “gold standard” for the non-invasive diagnosis of myocardial injury in human and animals, although in animals without inducing myocardial damage levels of cTnI and cTnT might undetectable (21). Of the cardiac troponin proteins, cTnI is more sensitive in most assays and is released into the circulation because of leakage from the damaged myocardial cells, in addition, Gunes *et al* (25), Peek *et al* (26), Leonardi *et al* (27), Suzuki *et al* (28) added that traumatic reticuloperitonitis, experimentally induced endotoxemia in cattle, and foot and mouth disease in calves have been reported to cause increased concentrations of circulating cTnI, same result of significant increase in cTnI was also detected in the current study, these results were also supported by the degeneration and necrosis of the myocardiocytes by the histopathologic sections of diseased

calves. Following myocardial damage in humans, cardiac troponins leak rapidly from the myocytes and appear in the blood after 2 to 4 hours and persist up to 10 to 21 days (29). Although the time course of troponin release in response to myocardial damage has not been determined exactly in cattle, a significant increase in serum or plasma cTnI concentration at one day after myocardial damage and a gradual reduction to physiological levels over the next 14 days occurs in sheep (27).

Another type of biomarkers is homocysteine Hcy which is a thiol-containing amino acid produced by the intracellular demethylation of methionine and exported into plasma where it circulates mostly in its oxidized forms bound to plasma proteins, furthermore, epidemiological studies have investigated the relationship between (Hcy) levels in blood and cardiovascular disease (30). It had been postulated that the association between serum homocysteine and myocarditis still not well defined (31), On the contrary it had been proven that the determination of serum Hcy concentration in calves with FMD might useful as a guide to quantify cardiac associated damage (32). Whereby, homocysteine is an intermediate amino acid in the methionine metabolism which is not present in proteins as hyperhomocysteinemia is an independent risk factor for cardiovascular diseases and had a role in generation oxidative damage (30). Significant increase of Hcy was detected in diseased calves in the present study, furthermore, it had been reported that plasma and heart tissue homocysteine were higher in the acute nutritional muscular dystrophy caused by vitamin E and selenium deficiency affecting the heart (33).

Comparing between cardiac biomarkers had been documented that, after cardiac injury started, serum CK-MB concentration will increase faster and peaked within 24 hour and serum LDH within 48 -72 hours ,whereas cardiac troponin starts to increase during the 3-4 hours and were peaked at the 12 hours, those results were confirm by the results of the present study and agreed with others (34), therefore, it were reported that CK-MB levels, as a marker of myocardial cell damage, are often increase in its range during the very early stage of myocarditis (17). Hereby the current study can be suggested that the stage of the disease is very early. It was indicated that lymphocytosis is one of the hematological features of the current study as significant increase of lymphocyte count were encountered in FMD infected calves, where lymphocytosis is indicated mostly in acute and active viral infection (35). A significant increase in the sedimentation rate of erythrocytes was also indicated in this study, since ESR were reflected the generalized inflammation as ESR rate will increase as a result of any cause of inflammation, whereby when an inflammatory process is present, Fibrinogen enters the blood in high amounts and causes red cells to stick to each other, which raises the ESR (36).

In the present study acute phase response was increased in diseased calves compared with controls, which, reflected by the significant increase in haptoglobin and fibrinogen. Acute phase response is the sum of the systemic and metabolic changes occurred by release of acute phase proteins in response to inflammatory stimulus, they are plasma proteins which increase or decrease in concentration following infection, inflammation or trauma, moreover they are a class of proteins whose plasma concentrations increase or decrease in response to inflammation, however, this type of response considered as a complex reaction, involving local and systemic effects, since one of these effects corresponds to changes in the concentration of some plasma proteins, basically synthesized in the liver, which are called acute phase proteins, Which is induced by cytokines acting as messengers between the local site of injury and the hepatocytes (37). Moreover, Cary *et al* (38) were also added that this response will stimulate to mobilize nutrients for the increased needs of the activated immune system, as well as for energy production and tissue repair.

Conclusion

It can be concluded that determination of cardiac biomarkers and acute phase response concentration in calves with myocarditis can considered as a guide to quantify early heart damage.

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