Evaluation of a Diagnostic Model for Aflatoxicosis in Sheep: A Prerequisite for Future Adoption of National Surveillances

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KEY WORDS: aflatoxicosis, aflatoxin B1, diagnostic model, evaluation, sheep

ABSTRACT

The aim of this research is to evaluate a diagnostic model for uncovering an aflatoxicosis outbreak in sheep. The diagnostic model is based on differentiation between an aflatoxicosis suspected herd (ASH) and a control herd (CH) including, clinical findings, feed analysis for aflatoxin and other nutrients, hemato-chemical and biochemical profiles, histopathologic changes, and residual aflatoxin levels in their organs. There was a significant difference between the sheep of the ASH and CH in relation to mortality percent, symptoms, aflatoxin B1 level in their feed and tissues, hematological and biochemical parameters, liver and kidney enzymes and metabolites, serum electrolytes, and vitamins A and E. The relationship of the histopathological lesions of affected

tissues to the aflatoxin B1 is discussed. This diagnostic model resulted in significant differences among many assigned parameters in ASH compared to the CH, allowing for its future adoption in national surveillances of aflatoxicosis in sheep.

INTRODUCTION

Aflatoxins are secondary metabolites produced by the fungus *Aspergillu.s*[1-3] The produced aflatoxins are acutely toxic, immunosuppressive, mutagenic, teratogenic, and carcinogenic compounds, targeting mainly the liver organ, inducing toxicity and carcinogenicity.[4]

In livestock, the consumption of aflatoxin-contaminated feed causes many health problems. Aflatoxin B1 (AFB1) acts as a hepatotoxicant, hepatocarcinogen and mutagen.[5] The acute toxic effects of AFB1 include hemorrhage and death. Chronic exposure to the aflatoxin affects growth rate, feed efficiency and susceptibility towards bacterial and viral associated diseases.[6] Lipid peroxidation and oxidative DNA damage are the principal manifestation of AFB1-induced toxicity, which could be mitigated by antioxidants.[7] Small amounts of AFB1 can cause mild or negligible effects, while large amounts cause serious damages in the host. Animals with lowest possible exposure to aflatoxins are those existing in a free range system. Low levels of aflatoxin may not be detected since they are excreted rapidly from the body.[8]

The basis of presumptive diagnosis of aflatoxicosis in sheep relies on observation of mortality, gross lesions on the mucosal layers, cyanosis, and petechial hemorrhage in the liver, associated with symptoms of weakness and diarrhea. It is of paramount importance to reach to final reliable diagnosis based on the hypothesis of inclusion of many other observations and analysis including hematological profiles, feed analysis for aflatoxin, serum biochemical and chemical profiles, gross and microscopic lesions, and residual level of aflatoxin in the organs of affected sheep.

The aim of this research is to evaluate a diagnostic model for aflatoxicosis, based on clinical findings, feed analysis for aflatoxin, hemato-chemical and biochemical profiles, histopathological changes, and residual aflatoxin in sheep organs of an ASH versus a CH, in an attempt to adopt the model in future regional surveillances.

MATERIAL AND METHODS

Herds

Two sheep herds of Awassi breed were included in this research, located at Bahran region of the subtropical kingdom of Saudi Arabia. The first herd consisted of 500 sheep, showing symptoms and gross lesions of aflatoxicosis, labeled as ”aflatoxicosis suspected herd” (ASH), and the other herd consisted of 100 sheep, showing no symptoms of any disease, labeled as ”control herd” (CH). Records of mortality in the two flocks were documented, and signs of the suspected condition were recorded.

Blood and Feed Sampling

Blood was collected from the jugular vein of the sheep in the two herds. The respective number of sampled sheep from the ASH and the CH were 50 and 10. Two blood samples were collected from each sheep, one is collected over heparin for hematological profiles while the other sample was collected for serum collection. Two feed samples were collected from each of the two herds, one from the feeder and the other from the stored feed, totaling to four samples.

Feed Analysis

Each of the four samples of feed was analyzed in duplicate for humidity, ash %, total protein %, ether extract % (fat), calcium (mg/dl), inorganic phosphorus (mg/dl), magnesium (mg/dl), potassium (mEg/l), sodium (mEg/l), aflatoxin B1 (ppb), and ochratoxin (ppb). The determination of aflatoxin B1 and ochratoxin by thin layer chromatography, and the proximate analysis of nutrients were accomplished as described by the Association of Official Agricultural Chemists[9].

Hemato-biochemical and Chemical Analyses of Blood

The hematological analysis applied on non-

coagulated blood samples included the counting of white blood cells, neutrophils, lymphocytes, monocytes, eosinophils, and basophils.[10] In addition, the RBC count, hemoglobin %, and PCV were determined by previously documented procedures.[11] The biochemical analysis of the collected sera included the analysis for alanine aminotransferase (ALT) and aspartate-aminotransferase (AST),[12] alkaline phosphatase (ALP),[13] total proteins (TP),[14] and albumin.[15] The determination of the globulin was calculated by subtracting the albumin level from the TP of the same serum sample. The serum creatinine and uric acid were determined by the respective previously documented methods.[16,17] The minerals in the blood (Ca, K, Mg, Na, and P) were determined by graphite furnace atomic absorption spectrometry,[18] while the vitamins A and vitamin E were determined by Thin-layer chromatography.

Table 1. *The means of ration composition*

Parameter	ASH[1] ration	CH[2] ration
Humidity %	8	7.8
Ash%	9	10
Total protein %	14	13
Ether extract %	4.3	4.1
Calcium (mg/dl)	2.1	2
Inorganic phosphorous (mg/dl)	1.3	1.2
Magnesium (mg/dl)	2	1.9
Potassium (mEq/l)	95	100
Sodium (Meq/l)	100	105
Aflatoxin B1(ppb)	300	Undetected level
Ochratoxin (ppb)	Undetected level	Undetected level

[1]ASH = Aflatoxin Suspected Herd
[2]CH = Control Herd

Organ Analysis for Aflatoxin B1 and Ochratoxin

Three nature of organs were collected from the ASH and CH sheep namely the liver, kidney, and muscle. The numbers of each organ from ASH versus CH were: liver (35 vs. 15), kidney (30 vs. 20), and muscles (12 vs. 38). The analysis for aflatoxin B1 and ochratoxin were according to the same procedures used for analysis of both toxins in the feed.

Histopathological study

The following number of samples were collected from the ASH vs the CH sheep namely, lungs (30 vs. 20), hearts (35 vs. 15), livers (35 vs. 15), kidneys (30 vs. 20), and intestines (30 vs. 20). Each sample was fixed in 10% neutral buffer formalin. The samples were processed by standard paraffin embedding technique, sectioned at a thickness of 5µm, stained with H&E procedure,19, and examined microscopically for recording the histopathological changes in these tissues.

Statistical analysis

The comparison of means of different measured parameters between the ASH and CH sheep was analyzed by t-student test using the SPSS statistical computing package (SPSS 14, 2006). Statistical differences were reported at P values of <0.001, <0.01, and <0.05.

RESULTS

Clinical findings

The clinical findings showed a 10% mortality in the aflatoxicosis-suspected herd (ASH) versus its absence in the control herd (CH) ($p<0.05$). The sheep in the ASH showed symptoms of weakness, depression, congestion of mucous membranes, and diarrhea. However, the control herd (CH) had a complete absence of these symptoms.

Feed analysis

The analysis of feed collected from the ASH versus that of the CH showed a similar composition of nutrients and humidity (Table 1). However, the aflatoxin B1 was high in the feed of the ASH (300 ppb) compared to the undetectable level in the feed of the CH. The

Table 2. *Mean values ± SE of erythrogram parameters of ASH[1] (N=50) and CH2 (N=10) sheep*

Herds	RBC(x10^6/µl)	Hb (g/dl)	PCV (%)	Mean Corpuscular Volume (MCV) (fL)	Mean Corpuscular Hemoglobin (MCH) (pg)	Mean Corpuscular Hemoglobin Concentration (MCHC)(g/dl)
CH	9.66 ±0.13	10.24 ±0.14^a	39.23 ±1.12^a	40.61 ±1.19	10.60 ±0.41	26.10 ±0.84
ASH	8.26 ±0.19	8.80 ±0.15^b	31.40 ±0.53^b	38.14 ±0.83	10.68 ±0.16	28.06 ±0.54

[1]ASH = Aflatoxin Suspected Herd
[2]CH = Control Herd
[a,b]Means in a column followed by different alphabet superscripts are significantly different at P<0.001

ochratoxin was undetectable in the feed of both ASH and CH.

Erythrogram and Leucogram Profiles

The profiles of the erythrogram and the leucogram are respectively shown in Tables 2 and 3. The mean values ± SE of the different parameters of the erythrogram showed significant lower red blood cells (RBC) counts, hemoglobin (Hb) %, and packed cell volume (PCV) in the ASH compared to controls (p<0.001) (Table 2). In addition, the mean values ±SE of the parameters included in the leucogram showed significant drop in the white blood cell (WBC) count of the ASH compared to CH (p<0.001) (Table 3). The other parameters of the erythrogram and leucogram did not differ significantly (p>0.05).

Hemato-biochemical and Chemical Analyses

The data related to hemato-biochemical analyses of sera, reflecting the liver and kidney functions of the ASH versus CH sheep, are shown in Table 4. Six out of the 10 parameters were elevated in the ASH compared to CH, with significant differences in ALT (p<0.001), AST (p<0.001), AP (p<0.001), LDH (p<0.01), urea (p<0.01), and creatinine (p<0.01). On the contrary, three other parameters were decreased significantly in the ASH compared to controls namely, the total protein (p<0.01), albumin (p<0.01), and globulin (p<0.01). It is worth noting that the ratio of the albumin/globulin did not differ significantly between the two herds (p>0.05).

The data related to the serum's chemical analysis of the sheep belonging to the two flocks are shown in Table 5. All chemical analysis of the seven measured serum parameters revealed a significant drop in sheep of the ASH compared to controls including, Ca (p<0.001), Pi (P<0.01), Mg (p<0.05), Na (p<0.05), K (p<0.05), Vitamin A (p<0.01), and Vitamin E (p<0.01).

Residual Aflatoxin B1 and Ochratoxin in Sheep Organs

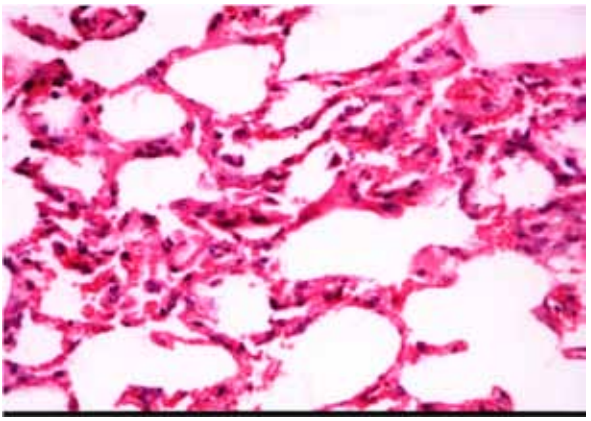
The range of aflatoxin B1 levels in livers, kidneys, and muscles of sheep in the compared two herds is shown in Table 6. All three organs of the ASH sheep showed higher range of aflatoxin B1 compared to levels in organs of the CH. The respective aflatoxin B1 ranges in sheep of ASH versus CH (µg/kg) were: livers (25-50 vs. 10-20), kidneys (45-70 vs. 30-40) and muscles (55-

Table 3. *Mean values ± SE of leucogram parameters (absolute values x10^3/µl) of ASH[1] (N=50) and CH[2] (N=10).*

Herds	WBCs	Neutrophils	lymphocytes	Monocytes	Eosinophils	Basophils
CH	8.76±0.18a	2.09 ±0.14	6.26 ±1.12	0.25 ±1.19	0.12 ±0.00	0.04 ±0.02
ASH	6.02±0.56b	1.92 ±0.34	3.74 ±0.53	0.21 ±0.02	0.10 ±0.02	0.04 ±0.02

[1]ASH = Aflatoxin Suspected Herd
[2]CH = Control Herd
[a,b]Means in a column followed by different alphabet superscripts are significantly different at P<0.001

Fig 1. *Lung of ASH sheep showing mild emphysema of alveoli, dilation of bronchi, with proliferation of epithelial cells lining bronchi associated with congestion of some pulmonary blood vessels, and presence of blood corpuscles hemolysis (H&E staining).*



80 vs. 40-80)

Histopathology

The histopathology of ASH sheep lungs (Fig 1), hearts (Fig 2), liver (Fig 3), kidney (Fig 4), and intestine (Fig 5) showed various microscopic changes, detailed in their legends, with predominance of congestion, hemorrhage, necrosis, and edema.

DISCUSSION

The clinical findings in the two compared herds of sheep, based on the presence in the first herd of 10% mortality and symptoms conforming with the aflatoxicosis, and the absence of mortality and symptoms in the control herd (CH), helped in the presumptive diagnosis of the affected animals as belonging to an "aflatoxicosis suspected herd" (ASH). It is worth noting that the LD50 of AFB1 in sheep is reported at 1.00-2.00 mg/kg body weight.[20]

The acceptance of the hypothesis that the designed diagnostic model in this research could lead to a final diagnosis of aflatoxicosis, depended on comparative analysis of a wide spectrum of parameters in the feed and samples collected from the sheep of the ASH and CH.

The analysis of the feed collected from the ASH versus that of the CH (Table 1) showed a similar composition of nutrients and humidity, thus eliminating differences in available nutrients offered to both flocks. However, the level of aflatoxin B1 in the feed of the ASH was higher (300 ppb) compared to the undetected level in the feed of the CH. The unacceptability of the 300 ppb level is based on standards reported previously for acceptable limit of aflatoxin B1 in the feed of dairy animals, which is set at 5 ppb.[21]

The non-coagulated blood collected from the sheep of ASH and CH, and subjected to the erythrogram (Table 2) and leucogram (Table 3) profiles revealed a significant drop in the RBC count of the ASH compared to CH sheep ($p<0.001$). This observation, within the diagnostic model, is in agreement with data presented by other workers,[22] suggesting that this decline in RBC is due to the aflatoxin effect on the myeloid tissue of the bone marrow.[23] In addition, the leucogram revealed a significant lower count in the WBC in the sheep of the ASH compared to that of the CH, which is most likely result-

Fig 2. *Heart of ASH sheep showing Zenker necrosis of most of cardiac muscles, severe oedema dispersing the cardiac muscles, mononuclear inflammatory cells, associated with hemorrhage in between the cardiac muscles (H&E staining).*

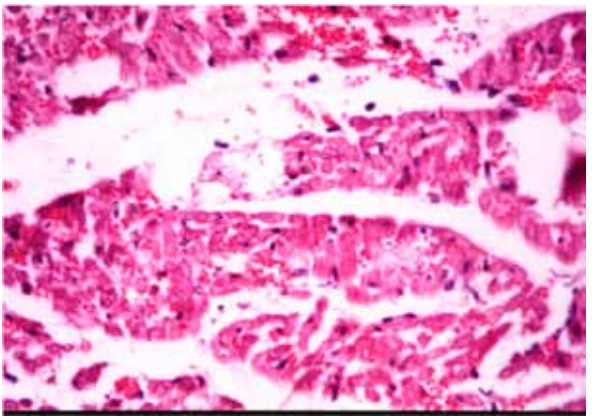


Table 4. *Means of liver and kidney parameters of ASH1 and CH2 sheep reflecting the two organs functions*

Blood biochemical parameters	ASH (N=50)	CH (N=10)
ALT (IU/ml)	95 ±1.6[a]	31 ±1.7[b]
AST(IU/ml)	92 ±1.3[a]	40 ±1.6[b]
ALP(U/L)	325 ±2.4[a]	139 ±1.9[b]
LDH(IU/l)	2500 ±5.6[c]	730 ±4.3[d]
Urea (mg/dl)	65 ±1.9[c]	29 ±1.1[d]
Creatinine (mg/dl)	1.88 ±0.03[c]	0.9 ±0.02[d]
Total protein (g/dl)	5.1 ±0.02[c]	7.5 ±0.09[d]
Albumin (g/dl)	2.8 ±0.01[c]	3.8 ±0.03[d]
Globulin (g/dl)	2.3 ±0.02[c]	3.7 ±0.02[d]
Albumin/Globulin ratio	1.2 ±0.01[e]	1.02 ±0.03[e]

[1]ASH = Aflatoxin Suspected Herd
[2]CH = Control Herd
[a,b]Means in a row followed by different alphabet superscripts of 'a' and 'b' are significantly different at P<0.001
[c,d]Means in a row followed by different alphabet superscripts of 'c' and 'd' are significantly different at P<0.01
[e]Means in a row followed by the same alphabet 'e' are non-significantly different at P>0.05

ing from the immunosuppressive effect of aflatoxin B1 present in the administered feed to sheep of the ASH. Previous researchers proved that a level of aflatoxin B1 equivalent to 7.2 µg/kg can induce leucocytopenia in rats,[24] a condition that is responsible for immunosuppression by this toxin.[25]

The compared hemato-biochemical and chemical analyses of the sheep sera of the ASH and CH (Table 4) resulted in significant rise of the four serum enzymes in the ASH sheep namely, the ALT, AST, ALP, and LDH, indicating an injury to the liver.[26] The injury to the kidney was confirmed by the significant rise in urea and creatinine level in the sera of ASH sheep, a result that is in agreement with previous published work on aflatoxin B1 in rabbits.[27] On the contrary, three other parameters in the serum declined significantly in the ASH sheep compared to the CH namely, the total protein, albumin, and globulin, which is most likely due to the injury by the toxin to the liver and the hemopoetic and lymphoid tissues.[28,29]

The consistent drop in all the analyzed serum levels of minerals and vitamins in the ASH sheep compared to the CH (Table 5) reflects the injury on the kidney by the ingested aflatoxin, resulting in loss of homeostasis of electrolyte balance,[30,31] and even vitamins A and E. The drop in serum vitamins A and E is most likely due to the use of these vitamins in their known

Table 5. *Means of serum minerals and vitamins of ASH1 (N=50) and CH2 (N=10) sheep*

Minerals & vitamins	ASH	CH
Total Calcium (mg/dl)	7.4±0.3[a]	11.2 ±0.92[b]
Inorganic Phosphorus (mg/dl)	4.1±0.61[c]	5.7 ±0.31[d]
Magnesium (mg/dl)	1.8±0.03[e]	3.1 ±0.11[f]
Sodium (mEq/l)	115±3.2[e]	135 ±2.44[f]
Potassium (mEq/l)	2.7±0.12[e]	3.8 ±0.09[f]
Vit. A (IU/dl)	21±0.94[c]	48 ±1.6[d]
Vit. E (µg/dl)	450±3.6[c]	715 ±3.9[d]

[1]ASH = Aflatoxin Suspected Herd
[2]CH = Control Herd
[a,b]Means in a row followed by different alphabet superscripts of 'a' and 'b' are significantly different at P<0.001
[c,d]Means in a row followed by different alphabet superscripts of 'c' and 'd' are significantly different at P<0.01
[e,f]Means in a row followed by different alphabet of 'e' and 'f' are significantly different at P<0.05

Fig 3. *Liver of ASH sheep showing necrosis of hepatocytes, vacuolar degenerative changes, oedema dispersing hepatocytes, and disorganization of liver cell arrangement (H&E staining)*

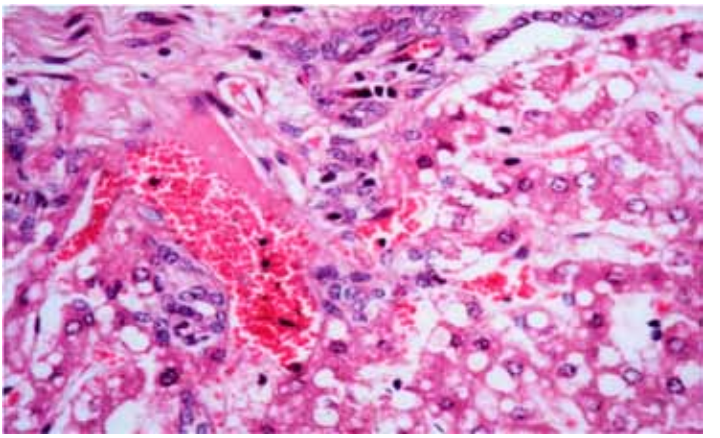


Fig 4. Kidney of ASH sheep showing periglomerular oedema, dispersing oedema of the renal tubes, vacuolar degeneration of epithelial cells lining the renal tubules, and necrosis (H&E staining).

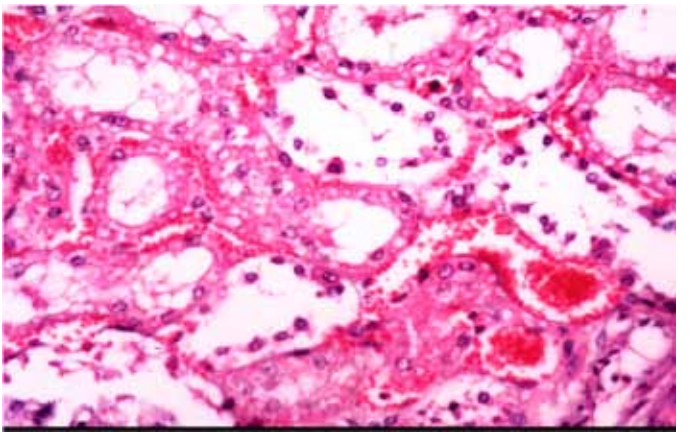
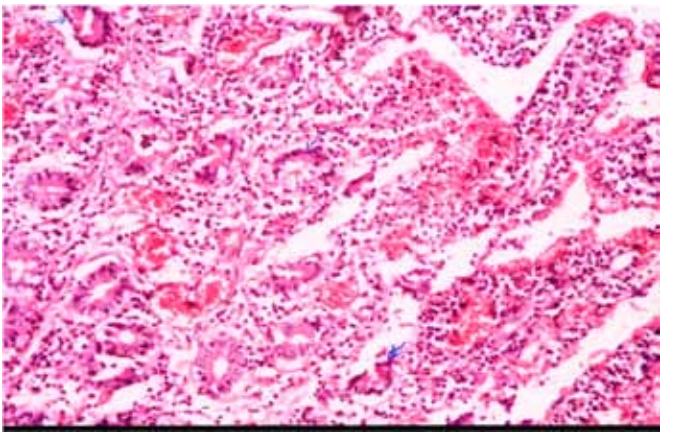


Fig 5. *Intestine of ASH sheep showing villar hemorrhage, infiltration of mononuclear inflammatory cells, associated with complete necrosis of intestinal glands (H&E staining).*



protective role against aflatoxin B1-induced oxidative stress.[32] This pattern of significant differences in the minerals and vitamins parameters strengthens the reliability on the diagnostic model put in the hypothesis of this research.

The sheep in CH had an acceptable range of the aflatoxin residue in their livers. However, all examined control sheep have an unacceptable level of this aflatoxin in their kidneys and muscles that were very near to the levels in these two organs of the ASH. The half-life of aflatoxin B1 in different organs of the sheep is not yet determined. Its presence in the CH sheep's kidney and muscle could be due to remote ingestion of aflatoxin.[33] Future investigation should study the half- life of this toxin in different sheep organs. The liver seems to reflect the present situation in which the ASH received aflatoxin contaminated feed, while the feed offered to controls had an undetectable level at the same time that this study was performed. The research in human body shows that the aflatoxin converts into AFQ1 by the cytochrome P450 enzyme (P450IIIAY), a process that is not yet proven to exist in kidneys and muscle fibers.[33]

The diagnostic model was further strengthened by the results of the histopathologic study, showing the absence of microscopic lesions from sheep of the CH and the presence of the specific aflatoxicosis lesions in different organs of the ASH. The sheep of the ASH had lungs with emphysema of alveoli, dilation of the bronchi and proliferation of their epithelial cells, congestion, and hemolysis of blood corpuscles (Fig. 1), observations that are not previously reported in sheep, but are extensively studied in rat and mouse models.[34]

The Zember necrosis in the heart of the ASH sheep, associated with edema and hemorrhage, are shown in Fig. 2. The liver necrosis of the hepatocytes associated with edema and vaculosis (Fig. 3), the periglomerular oedema, necrosis, and degeneration of the epithelial cells lining of the renal tubules (Fig. 4), the presence of villous hemorrhages associated with mononuclear inflammatory cells infiltration and complete necrosis of some intestinal glands (Fig. 5) are collective new microscopic observations, that could indicate the involvement of other targeted organs of sheep by the injuries created by aflatoxin B1.

CONCLUSION

The implementation of the diagnostic model on the ASH and CH sheep resulted in data that confirmed the reliability of the applicability of this model to reach to a final diagnosis of aflatoxicosis in sheep. The many agreements between the obtained data and those reported in literature and the significant differences in many analyzed parameters between the sheep of the ASH and CH, allows for accepting the hypothesis of implementing this model for reaching to a final diagnosis of sheep aflatoxicosis. It is recommended in the near future to include this diagnostic model in the national surveillances of aflatoxicosis in sheep of the subtropical areas of the Middle Eastern countries, and other regions of the world that are experiencing similar signs in their aflatoxicosis suspected herds.

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