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Accidental fatal aflatoxicosis due to contaminated commercial diet in 50 dogs

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ABSTRACT

Aflatoxins, produced by *Aspergillus* spp., are toxic contaminants of stored grain. This study describes 50 dogs presented with foodborne aflatoxicosis. Common clinical signs included lethargy (78%), vomiting (76%), anorexia (74%), icterus (66%), depression (66%), melena (60%), haematuria (36%) and diarrhoea (36%). Common laboratory abnormalities included increased activities of aspartate aminotransferase (86%), alkaline phosphatase (84%) and alanine aminotransferase (79%), hypoantithrombinaemia (86%), prolonged prothrombin (PT, 82%) and activated partial thromboplastin times (aPTT, 80%), hyperbilirubinaemia (73%), hypocholesterolaemia (60%) hypoalbuminemia (47%) and thrombocytopenia (42%). Non-survivors had longer PT and aPTT and lower antithrombin ($P < 0.001$) at presentation compared to survivors (23.8 s *vs.*10.5; 37.9 *vs.*17.6s and 5% *vs.* 54%, respectively). Hyperbilirubinaemia (>56.6 μmol/L) and albumin concentration <32.5 g/L at presentation were risk factors for mortality ($P < 0.0001$). Common complications included disseminated intravascular coagulation (58%), hepatic encephalopathy (35%) and acute kidney injury (4%). The mortality rate was 68%, suggesting that dogs with aflatoxicosis have poor prognosis.

1. Introduction

Aflatoxins are a group of toxic compounds, produced by several *Aspergillus* spp. fungi, mainly *A. flavus* and *A. parasiticus*. Fungal growth and toxin production occurs under warm environmental temperatures, mostly in tropical and subtropical climates (Puschner, 2002; Williams et al., 2004). Aflatoxins can be produced by fungi during harvest, storage, production and processing of cereals (e.g., corn) peanuts, cottonseed and tree-nuts. Because aflatoxins are extremely heat-stable, they might persist in processed foods that are no longer moldy (Williams et al., 2004), and are considered by the United States Food and Drug Administration unavoidable food contaminants (Puschner, 2002; Campos et al., 2008; Dereszynski et al., 2008). Aflatoxin has previously been detected in samples of most commercial domestic pet foods, including canine diets (Scudamore et al., 1997; Maia and Pereira Bastos de Siqueira, 2002; Leung et al., 2006; Stenske et al., 2006; Campos et al., 2008; Dereszynski et al., 2008).

Although many different aflatoxin types exist, aflatoxin B1 (AFB1) is the most hepatotoxic, carcinogenic and mutagenic of all food-borne related aflatoxins; however, its toxicity varies among animal species, with poultry and dogs being the most sensitive (Newberne and Butler, 1969; Bingham et al., 2004; Stenske et al., 2006; Dereszynski et al., 2008). Dogs are considered highly sensitive to aflatoxin, partly, due to their inherent, relatively lower, hepatocellular glutathione levels, and the reported aflatoxin lethal dose 50% is 0.5–1 mg/kg (Chaffee et al., 1969; Ketterer et al., 1975). Aflatoxin B1 undergoes hepatic biotransformation (phase I metabolism), mediated by cytochrome P450 oxyreductases to its active, electrophilic metabolite, AFB1-8-9-epoxide (AFBO). The latter, undergoes further metabolism (i.e., epoxidation), including phase-II glutathione conjugation (mediated by glutathione S-transferase), and is then eliminated through the bile or the kidneys (Zimmerman, 1999; Tulayakul et al., 2005). The reactive, unstable, intermediate metabolite, AFBO, may covalently bind to intracellular macromolecules, including structural proteins, enzymes, DNA and RNA, leading to disruption of basic cellular metabolism, protein synthesis and DNA repair and replication (Newberne and Butler, 1969; Puschner, 2002; Leung et al., 2006; Dereszynski et al., 2008). Hepatocellular toxicity and necrosis are directly induced by AFBO, as well as by secondary glutathione depletion, resulting in further decrease of hepatic aflatoxin detoxification, thereby exacerbating oxidative hepatocyte injury.

Aflatoxicosis can be acute, sub-acute or chronic, depending on the toxin amount consumed, the exposure period and species-specific susceptibility (Newberne and Butler, 1969; Greene et al., 1977; Bastianello et al., 1987). In people and farm animals, chronic aflatoxicosis occurs more commonly, mostly in developing countries (Chaffee et al., 1969; Greene et al., 1977; Scudamore et al., 1997; Williams et al., 2004; Muture and Ogana, 2005). Chronic aflatoxicosis is predominantly perceived as a promoter of liver cancer and compromised immunity (Williams et al., 2004). In contrast, in dogs, acute and sub-acute aflatoxicosis have been the most frequently reported forms of this poisoning, and mostly resulted in

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Table 1
Risk factors for mortality in 50 dogs with naturally-acquired foodborne aflatoxicosis.

Variable	Survivors Number of dogs (%)	Non-survivors Number of dogs (%)	OR[a] (CI_{95})[b]	*P* value
Icterus	3/16 (18)	27/34 (79)	16.7 (3.7–76.9)	<0.0001
Neurological signs	1/16 (6)	20/34 (58)	21.2 (2.5–166.7)	0.001
Melena	3/16 (19)	23/34 (67)	9.1 (19.4–38.5)	0.002
Haematuria	0/16 (0)	15/34 (44)	NA	0.001
Disseminated intravascular coagulation	3/16 (19)	25/34 (74)	8.3 (2.1–32.3)	0.002
Prothrombin time ⩾14.95 s	4/16 (25)	29/34 (85)	19.6 (4.0–76.9)	<0.0001
aPTT[c] ⩾25.65	2/16 (13)	25/34 (74)	19.6 (3.7–100.0)	<0.0001
Antithrombin activity ⩽17.5%	2/15 (13)	17/22 (77)	22.2 (3.7–125.0)	<0.0001
Serum albumin ⩽32.5 g/L	1/14 (7)	24/30 (80)	52.6 (5.6–500.0)	<0.0001
Serum total bilirubin ⩾56.6 μmol/L	1/14 (7)	23/31 (74)	37.0 (4.2–333.3)	<0.0001
Serum cholesterol ⩽2.75 mmol/L	3/14 (21)	21/29 (72)	9.6 (2.1–43.8)	0.003

[a] OR – odds ratio.
[b] CI_{95} – 95% confidence interval.
[c] aPTT – activated partial thromboplastin time.

hepatic failure (Chaffee et al., 1969; Greene et al., 1977; Scudamore et al., 1997).

The most common clinical signs of acute aflatoxicosis in humans and dogs include icterus, haematemesis, haematochezia, diffuse haemorrhage and ascites, as well as signs associated with disseminated intravascular coagulation (DIC) (Chaffee et al., 1969; Greene et al., 1977; Scudamore et al., 1997; Muture and Ogana, 2005). Necropsy of eight dogs with fatal acute and sub-acute foodborne aflatoxicosis showed hepatomegally and dull yellow liver (Bingham et al., 2004). Hepatic histopathology revealed hepatocellular fatty degeneration, portal fibroplasia, necrosis, regeneration and biliary hyperplasia; while chronic exposure was characterised by marked lobular atrophy, bridging portal fibrosis, and regenerative hepatocellular nodules (Bastianello et al., 1987; Stenske et al., 2006; Newman et al., 2007). Other post mortem abnormalities included widespread infracts and diffuse bleeding and haemorraghic necrosis, which are characteristic of DIC (Greene et al., 1977).

Since 1975, sporadic cases and outbreaks of foodborne aflatoxicosis have been documented in dogs (Chaffee et al., 1969; Greene et al., 1977; Bastianello et al., 1987; Scudamore et al., 1997; Newman et al., 2007). The two most recent ones (in 1998 and 2005), both in the USA, were the most widespread outbreaks, and have occurred due to foodborne aflatoxin contamination of moldy corn in commercial canine diets (Stenske et al., 2006; Dereszynski et al., 2008). Recall instructions were immediately issued, however, mortality rate was high 64% (46 of 72 dogs) (Dereszynski et al., 2008; Diamond-pet-food-recall, 2010; FDA-report, 2010).

The present retrospective study describes the history, clinical signs, laboratory abnormalities, disease progression, treatment and outcome of 50 dogs with aflatoxicosis, affected during an outbreak that has occurred from December 2005 to February 2006 in Israel due to exposure to a contaminated commercial canine diet, and provides assessment of risk factors for death.

2. Material and methods

2.1. Selection of cases

Between December 2005 to February 2006, 50 dogs were presented to the Hebrew University Veterinary Teaching Hospital (HUVTH) and diagnosed with aflatoxicosis based on a history of consumption of an aflatoxin-contaminated commercial canine diet (Nutra Nuggets, Diamond Pet Foods, USA) that has resulted in clinical signs of disease and/or increased liver enzymes activity and/or coagulation abnormalities. The particular batches of this diet brand were determined to be contaminated with aflatoxin by the manufacturer, and a recall warning was issued by both the manufacturer and its Israeli distributer. In addition, 10 food samples of these batches, brought by dog owners, were analysed by the Israeli authorities, and their aflatoxin concentration ranged between 80 and 300 ppb, while an upper concentration limit of 20 ppb is permitted the US Food and Drug Agency regulations (Stenske et al., 2006). The Institutional Animal Care and Use Committee, HUVTH has exempted this retrospective study from review.

2.2. Definition of secondary complications and the outcome

Disseminated intravascular coagulation was diagnosed if dogs presented thrombocytopenia (<150 × 10^9/L) and two of the following: prolongation (>25%) of prothrombin and activated partial thromboplastin times (PT and aPTT, respectively), decreased (<80%) antithrombin activity (ATA, reference interval [RI] 87–140%) and clinical signs of bleeding (i.e., petechiae, echimoses, haematochezia, melena, haematemesis and haematuria). Hepatic encephalopathy (HE) was diagnosed when plasma ammonia concentration was above 140 μmol/L and diffuse central nervous system (CNS) signs were present.

Non-survivors included dogs that died naturally or were euthanased at their owners' request during hospitalisation, and within two months post discharge from the hospital due to severe clinical deterioration and a grave prognosis. Financial constrains did not play a role in this decision, since all the cost of therapy were met by the Israeli distributor of the contaminated diet.

2.3. Laboratory tests

Blood samples for complete blood count (CBC), serum chemistry and coagulation tests were collected from all dogs at presentation and every 48 h during hospitalisation. Whole blood samples for CBC were collected in potassium-EDTA tubes and analysed using automatic blood impedance analysers calibrated for canine blood (Abacus or Arcus, Diatron, Austria). Blood for serum biochemistry was collected in plain tubes, and sera were either analysed immediately or refrigerated (4 °C) pending analysis, performed within 24 h from collection using a wet chemistry autoanalyser (Cobas-Mira, Roche, Switzerland, at 37 °C). Blood samples for ammonia were collected in lithium-heparin vacuum tubes. Plasma was separated within 5 min from collection by centrifugation and analysed immediately using the above mentioned autoanalyser. Electrolytes were analysed using ion-selective electrode electrolyte analysers (OmniC or Omni, Roche, Germany). Blood samples for coagulation tests (PT, aPTT, fibrinogen and ATA) were collected in 3.2% trisodium-citrate tubes, centrifuged immediately and plasma was analysed within 30 min from collection (ACL 200, Instrumentation Laboratory, UK or KC-1 micro, Amelung, Germany).

Table 2
Haematology, coagulation and serum biochemistry results at presentation in 50 dogs with naturally-acquired foodborne aflatoxicosis.

Parameter	Survivors				Non-survivors				*P* value	RI[a]
	n	Median (range)	% > RI[a]	% < RI[a]	*n*	Median (range)	% > RI[a]	% < RI[a]		
WBC [b] ($\times 10^9$/L)	16	9.3 (5.4–22.9)	12	6	34	13.1 (6.2–84.3)	47	0	0.002	6.0–14.0
Hematocrit (%)	16	53.8 (29.0–62.4)	37	13	34	53.0 (12.9–65.8)	29	1	0.693	37.0–56.0
Platelets ($\times 10^9$/L)	16	193 (79–793)	6	31	34	171 (8–432)	0	47	0.114	150–700
Prothrombin time (s)	16	10.5 (5.1–42.0)	68	6	34	23.8 (4.5–100.0)	85	8	0.001	6.0–8.4
aPTT[c] (s)	16	17.6 (9.9–100.0)	50	13	34	37.9 (9.6–100.0)	94	3	<0.001	11.0–17.4
Antithrombin (%))0)activity)	15	54.0 (5.0–150.0)	20	66	22	5.0 (0.0–147.0)	5	95	<0.001	⩾80
Albumin (g/L)	14	36.0 (29.0–45.0)	28	0	30	26.0 (11.0–39.0)	2	70	<0.001	28.3–38.3
Alkaline phosphatase (U/L)	14	179 (65–1708)	64	0	31	480 (78–1910)	93	0	0.004	4–140
ALT[d] (U/L)	15	228 (33–889)	67	0	33	297 (6–1739)	96	0	0.097	5–103
Amylase (U/L)	11	600 (403–1079)	0	0	29	419 (83–974)	0	7	0.007	255–1270
AST[e] (U/L)	13	67 (45–259)	69	0	31	138 (15–748)	90	0	0.010	9–47
Bilirubin (μmol/L)	14	6.7 (1.2–43.7)	35	0	312	128.2 (5.4–297.5)	90	0	<0.001	2–4
Calcium (mmol/L)	12	2.6 (2.0–3.2)	33	0	29	2.4 (0.47–3.1)	10	51	0.008	2.2–2.5
Cholesterol (mmol/L)	14	3.6 (1.5–16.8)	14	28	29	0.9 (0.23–7.9)	0	72	0.005	2.58–5.85
Creatine kinase (U/L)	12	134 (50–452)	33	0	29	118 (27–516)	24	0	0.431	13–250
Chloride (mmol/L)	7	110.3 (106.0–120.3)	14	0	24	102 (104–115)	4	75	0.009	102–117
CO_2 (mmol/L)	8	34.5 (26.0–36.0)	75	0	25	25 (11.0–44)	24	56	0.114	26–32
Creatinine (μmol/L)	15	97.2 (54.8–777.9)	20	0	31	618.0 (8.8–884.0)	15	22	0.079	50–110
Globulins (g/L)	13	30.0 (18.0–40.0)	0	15	28	27 (14–42)	25	18	0.935	0–6
GGT[f] (U/L)	13	6.4 (0.0–30.9)	23	0	30	8.7 (0.0–44.4)	20	0	0.947	0.0–19.0
Glucose (mmol/L)	13	5.25 (4.65–5.6)	46	0	28	5.3 (10–8.25)	50	3	0.657	3.9–6.1
Potassium (mmol/L)	7	4.18 (3.53–4.50)	0	28	24	3.98 (3.10–5.70)	4	37	0.560	3.80–5.60
Sodium (mmol/L)	7	155.5 (123.6–157.0)	43	14	24	151 (123–183)	25	8	0.178	140–154
Phosphorus (mmol/L)	12	1.19 (0.9–4.0)	16	0	27	1.5 (0.09–4.8)	15	11	0.060	0.8–1.6
Total protein (g/L)	13	67.0 (56.0–81.0)	8	8	30	52.0 (1.1–7.9)	7	50	<0.000	54.0–71.0
Triglycerides (mmol/L)	12	0.6 (0.4–1.5)	8	8	27	0.4 (1.4–2.7)	15	4	0.039	0.1–5.6
Urea (mmol/L)	13	8.7 (5.3–11.9)	8	0	29	9.9 (0.9–12.3)	17	2	0.634	7.2–14.2
Ammonia[g] (μmol/L)	7	141.8 (70.9–195.0)	100	0	20	165.8 (5.0–345.0)	88	0	0.439	5.9–80.0

* Significant ($P \leqslant 0.05$) difference between groups.
[a] RI – reference interval.
[b] WBC – White Blood Cells.
[c] PTT – activated partial thromboplastin time.
[d] ALT – Alanine aminotransferase.
[e] AST – Alanine aminotransferase.
[f] GGT – γ-glutamyl transferase.
[g] Maximal recorded value during hospitalisation.

2.4. Statistical methods

Shapiro–Wilk's test was used to assess the normality of data distribution of continuous parameters. Continuous parameters are reported as median and range, and were compared between survivors and non-survivors using the Mann–Whitney *U*-test. Fisher's exact test was used to compare categorical variables between outcome groups. Assessment of breed as a risk factor for developing aflatoxicosis was performed by comparing breed frequencies between the study population and the general HUVTH population (years 1999–2004). Repeated measures ANOVA were used to assess changes in continuous variables over time, and to compare the differences between outcome groups. Correlations between laboratory analytes were assessed using Spearman's Rank correlation test. The association between continuous parameters and the outcome was evaluated by the receiver operator characteristics (ROC) procedure, calculating of the area under the curve (AUC) with its 95% confidence interval ($CI_{95\%}$). Selected optimal cutoff points with their corresponding sensitivity and specificity for prediction of the outcome were those with the least number of misclassifications. Because dogs were presented at different time intervals from the initial exposure to the contaminated diet, and in order to eliminate the variance introduced by such different time-lags, the analyte levels used in the risk factor analysis included both their levels at presentation and the most extreme deviation from reference interval (RI) observed during hospitalisation.

For all tests, a *P*-value $\leqslant 0.05$ was considered statistically significant. All calculations were performed using statistical software (SPSS 17.0, SPSS Inc. USA).

3. Results

3.1. Signalment and duration of consumption of the aflatoxin-contaminated diet

Fifty dogs (25 males and 25 females) met the inclusion criteria and were included in this study, with a median age of 5 years (range, 0.75–11) and a median body weight of 26.5 kg (range, 2.2–39.0), with no age and body weight differences between survivors and non-survivors. Dog breeds included mixed-breed (21 dogs), golden and Labrador retriever (6), German shepherd (4), Staffordshire bullterrier, American Staffordshire and Pit Bull Terriers (7), boxer (3), Yorkshire terrier (3), rottweiler (2), Rhodesian ridgeback, Alaskan malamute, Akita, Shar-pei and Belgian malinois (1 each). Small breeds (<15 kg) were significantly underrepresented compared to the general HUVTH population (6% *vs.* 19%; odds ratio [OR] 0.32, $CI_{95\%}$, 0.08–0.95) ($P = 0.02$).

The median consumption time-period of the aflatoxin-contaminated diet of 22 dogs in which these data were available was 45 days (range 14–60), with no difference between non-survivors and survivors.

3.2. Clinical signs

Seven dogs (14%) were clinically normal at presentation, and were presented either following the alert issued in the media or because another dog in the household had been previously diagnosed with aflatoxicosis. All these seven dogs had laboratory abnormalities at presentation and did eventually develop clinical

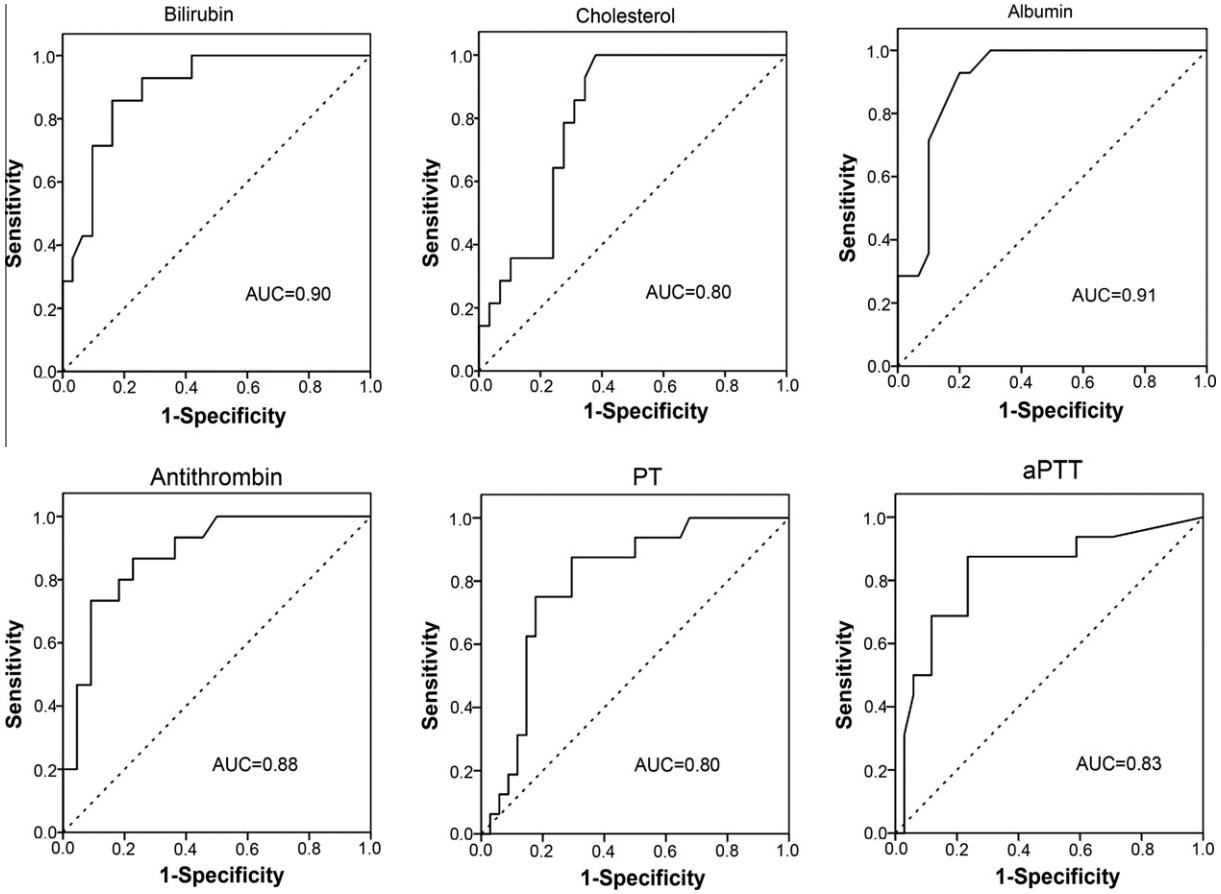

Fig. 1. Receiver operating characteristics (ROC) curves of coagulation and liver function analytes at presentation in dogs with aflatoxicosis, as predictors of the mortality. The area under the ROC curves ranges between 0.80 and 0.91, suggesting good performance of these analytes as predictors of the outcome.

signs during hospitalisation. The most common clinical signs, in the history and physical examination at presentation, included lethargy (78%), vomiting (76%), anorexia (74%), icterus (66%), depression (66%), melena (60%), haematuria (36%) and diarrhoea (36%). Five dogs developed peripheral edema during hospitalisation. Neurological abnormalities (i.e., severe depression, altered behaviour, vocalisation, seizures, stupor and coma) were observed during hospitalisation in 21/50 dogs and were significantly more common among non-survivors compared to survivors (20/34; 58.8% *vs*. 1/16; 6.3%, respectively) ($P = 0.01$). Anorexia, icterus, melena and haematuria were also significantly more common among non-survivors compared to survivors (Table 1).

3.3. Haematology and coagulation tests

The most common haematological and coagulation abnormalities at presentation were hypoantithrombinaemia (31/36 dogs, 86%), prolonged PT (41/50; 82%) and aPTT (40/50; 80%), thrombocytopenia (21/50; 42%) and leucocytosis (18/50; 36%). Median PT and aPTT at presentation were significantly longer in non-survivors compared to survivors (21.8 *vs*. 10.2 s, respectively, and 35.4 *vs*. 13.9 s, respectively, Table 2) ($P < 0.001$). ROC curve analysis of PT and aPTT at presentation as predictors of mortality yielded optimal cutoff points of 14.95 (AUC 0.80; $CI_{95\%}$ 0.67–0.92; sensitivity 75%; specificity 82%) and 25.65 s (AUC 0.83; $CI_{95\%}$ 0.69–0.96; sensitivity 88%; specificity 74%), respectively (Fig. 1). Dogs with PT $\geqslant$ 14.95 s and aPTT $\geqslant$ 25.65 s had a significantly higher mortality rate compared to those with PT and aPTT below these values (19% *vs*. 79% and 12.5% *vs*. 74%, respectively) ($P = 0.001$). Assessment of the changes of PT during hospitalisation showed a significant difference between survivors and non-survivors ($P = 0.012$), but there was neither a significant difference over time, nor was there an interaction between outcome groups (Fig. 2). In a similar analysis for aPTT, there was significant aPTT decrease over time in both groups ($P = 0.05$) and a significant difference between outcome groups ($P = 0.019$), with a consistently longer aPTT in non-survivors compared to survivors, but there was no interaction between groups (Fig. 2).

3.4. Serum biochemistry results

The most common serum biochemistry abnormalities at presentation (Table 2) were increased activities of aspartate aminotransferase (AST, 38/44; 86%), alkaline phosphatase (ALP, 38/45 dogs; 84%), and alanine aminotransferase (ALT, 37/48; 77%). Median ALP and AST activity were significantly higher in non-survivors compared to survivors ($P < 0.01$) (Table 2). There was no difference in ALT activity at presentation between non-survivors and survivors ($P = 0.31$), but the median highest recorded ALT activity in each dog during hospitalisation was significantly higher in non-survivors compared to survivors (367 *vs*. 272 U/L respectively) ($P = 0.05$). There were no correlations between serum CK activity versus serum AST and ALT activities.

Median serum cholesterol concentration was significantly lower in non-survivors compared to survivors, at presentation ($P = 0.005$) (Table 2) and throughout hospitalisation (Fig. 3). Median serum albumin concentration at presentation was significantly lower in non-survivors compared to the survivors ($P > 0.0001$) (Table 2). ROC curve analysis of serum albumin as a predictor of mortality yielded an AUC of 0.91 ($CI_{95\%}$ 0.82–0.99) with an optimal cutoff point of 32.5 g/L, corresponding to sensitivity and specificity of 93% and

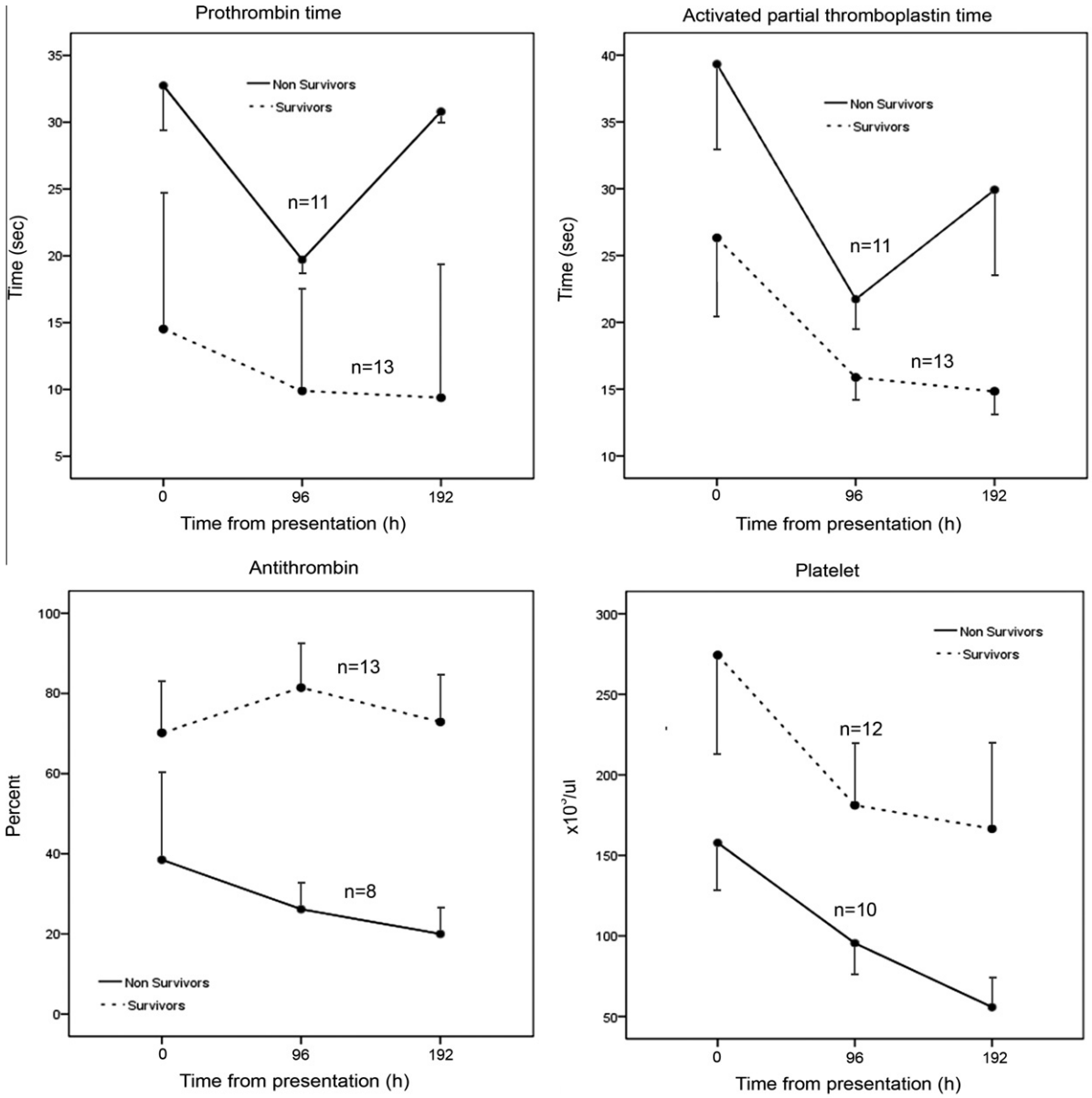

Fig. 2. Changes in coagulation analytes of dogs with aflatoxicosis over time, during hospitalisation. Dogs included in these analyses are those in which test results of all time-points during hospitalisation were available. Data are presented as mean and standard error. Note that all coagulation analytes are more severely deranged over time in the non-survivors compared to the survivors. Partial improvement of these analytes is noted in both survivors and non-survivors at 96 h of hospitalisation. However, while improvement in the survivors continued, the response was only transient in the non-survivors, despite fresh frozen plasma therapy. There was a significant change in platelet number over time ($P < 0.001$), with significant group differences ($P = 0.05$) and no significant interaction between groups. There was a significant difference in PT between survivors and non-survivors ($P = 0.012$), but there were neither a significant difference over time, nor an interaction between the outcome groups. There was significant aPTT decrease over time in both groups ($P = 0.05$) and a significant difference between outcome groups ($P = 0.019$), but no interaction between groups. There was a significant difference in ATA between non-survivors and survivors ($P = 0.027$), but there were neither a consistent change over time, nor was there an interaction between groups.

80%, respectively (Fig. 1). Analysis of serum albumin concentration changes during hospitalisation showed a significant difference between survivors and non-survivors ($P < 0.0001$), with the latter having consistently lower albumin concentration, but there were no significant change in albumin concentration over time nor was there an interaction between groups (Fig. 3).

Median serum bilirubin concentration at presentation was significantly higher in non-survivors compared to survivors ($P < 0.0001$) (Table 2). ROC curve of serum bilirubin concentration at presentation had an AUC of 0.90 ($CI_{95\%}$ 0.80–0.99) with an optimal cutoff point of 56.6 μmol/L, corresponding to sensitivity and specificity of 93% and 74%, respectively (Fig. 1). Dogs with bilirubin concentration >56.6 μmol/L at presentation had significantly higher mortality rate compared to those below it (96% *vs.* 38%, respectively; OR 37.04; $CI_{95\%}$ 4.2–393.3; $P < 0.0001$) (Table 1). During hospitalisation, there was a significant interaction between survivors and non-survivors ($P = 0.02$), with the former having consistently lower serum bilirubin concentration over time compared with the latter (Fig. 3). Median maximum plasma ammonia concentrations during hospitalisation were 141.8 and 165.8 μmol/L in survivors and non-survivors respectively, with no significant difference between groups ($P = 0.439$) (Table 2). Serum lactate, mea-

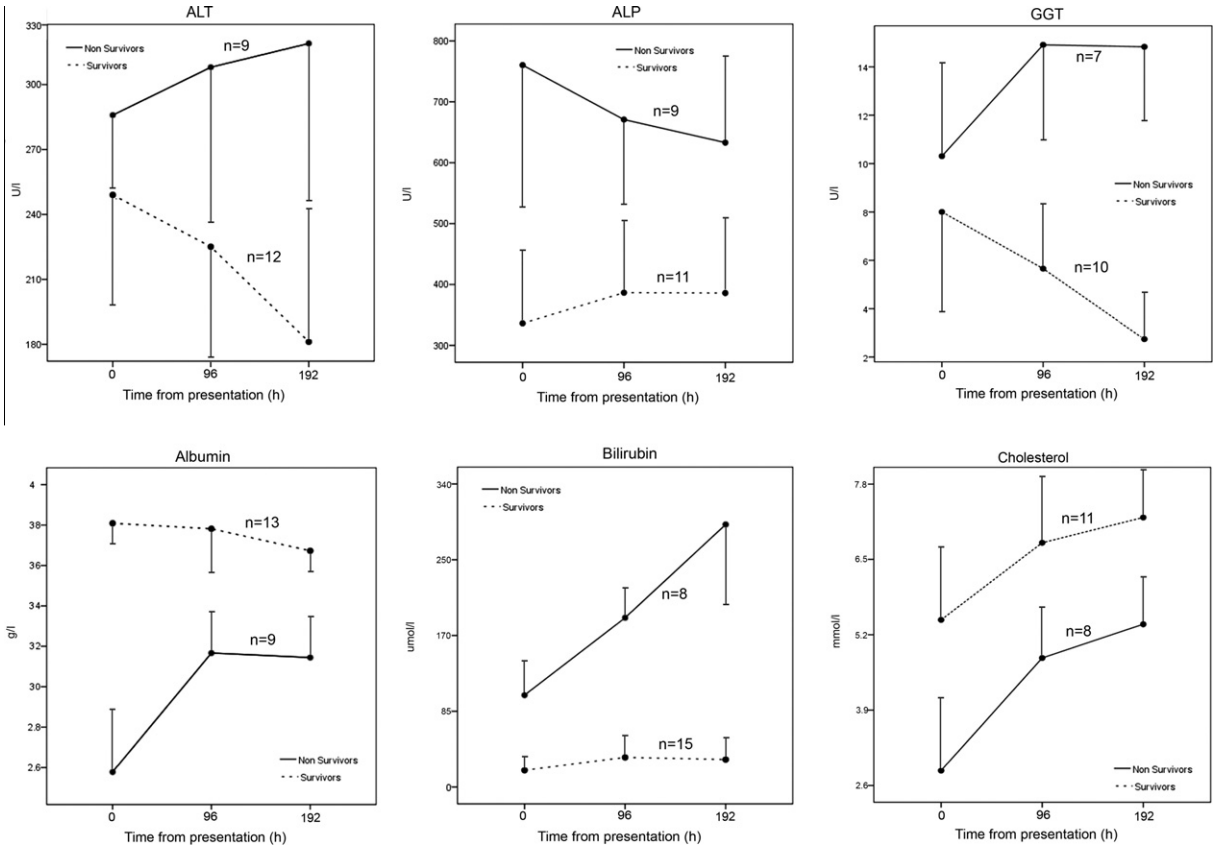

Fig. 3. Changes in activity of selected hepatobilliary enzyme activity and analytes of liver function of dogs with aflatoxicosis over time, during hospitalisation. Dogs included in these analyses are those in which test results of all time-points during hospitalisation were available Data are presented as mean and standard error. Note that activities of all enzymes are higher in the non-survivors compared to the survivors while abnormalities in analytes of liver function are more severe in the latter group throughout hospitalisation. There were no statistically significant changes in ALT and ALP activity over time, and any statistically significant difference or interaction between groups. There was a significant group interaction in GGT activity ($P = 0.046$). There was a significant difference in albumin concentration between survivors and non-survivors ($P < 0.0001$), but no significant change in albumin concentration over time or an interaction ($P = 0.088$) between groups. There was a significant group interaction in bilirubin concentration ($P = 0.02$). There was statistically significant changes in cholesterol concentration over time ($P = 0.023$), but there were no group differences ($P = 0.14$) or interaction.

sured in eight dogs at presentation (median 3.25 mmol/L, range 1.37–5.66) was above RI (>2.8 mmol/L) in five dogs, with no difference between outcome groups.

3.5. Associations between laboratory analytes

There was a significant positive correlation between aPTT and serum aspartate aminotransferase (AST) activity and bilirubin concentration ($P = 0.0001$; $r = 0.55$ and $r = 0.59$, respectively), and negative correlation with concentrations of serum albumin ($P = 0.006$; $r = -0.41$) and cholesterol ($P = 0.004$; $r = -0.44$) at presentation. PT at presentation was significantly and positively correlated with serum AST activity ($P = 0.002$; $r = 0.45$) and bilirubin concentration ($P = 0.001$; $r = 0.53$), but not with serum albumin and cholesterol levels.

At presentation, median ATA was significantly lower in non-survivors (5%; range 0.0–147%) compared to survivors (54%; range 1.4–150%; $P < 0.001$) (Table 2). Analysis of changes in ATA over time during hospitalisation showed a significant difference between non-survivors and survivors ($P = 0.027$), but there were neither a consistent change over time, nor was there an interaction between groups (Fig. 2). At presentation, there were significant positive correlations between ATA and serum albumin ($P = 0.0001$; $r = 0.56$) and cholesterol concentrations ($P = 0.004$; $r = 0.48$) and negative correlations with serum AST activity ($P = 0.0001$; $r = -0.61$) and bilirubin level ($P = 0.0001$; $r = -0.72$). Both aPTT and PT were significantly and negatively correlated with ATA ($P = 0.0001$; $r = -0.66$ and $r = -0.64$, respectively).

3.6. Abdominal ultrasonography

Abdominal ultrasound, performed in 22 of 50 dogs, most commonly showed hepatic hyperechogenicity (11/22 dogs, 50%), hepatomegaly (6/22, 27%) and abdominal effusion (5/22, 23%), distended gall bladder with thickened wall (>3 mm, 5/22, 23%) with no significant differences in their occurrence between non-survivors and survivors ($P = 0.32$ and $P = 0.097$, respectively).

3.7. Treatment

Treatment was essentially symptomatic and supportive, and its details and indications are provided in Table 3. Fresh frozen plasma (FFP) was administrated during hospitalisation to all dogs but one (a total of 736 units, approximately 250 ml/unit), at 40 ml/kg/d IV, with a median of 12.2 units per dog (range 0–40), corresponding to a median dose of 216 ml/kg (range 0–711) with no significant difference between survivors and non-survivors (median 313 ml/kg; range 0–711 and median 208; range 0–605, respectively, $P = 0.17$).

3.8. Hospitalisation, complications and outcome

The median hospitalisation time-period of all dogs was 6 days (range 0–21). Survivors had a longer, albeit insignificant ($P = 0.08$), hospitalisation period, compared to non-survivors (median 9.5 days; range 0–20 *vs.* median 5; range 0–21, respectively).

DIC was the most common documented complication (29/50 dogs; 58%), and was significantly more common in non-survivors

Table 3
Drugs with doses, dosing intervals, routes of administration and indications used in the treatment of 50 dogs with naturally-acquired foodborne aflatoxicosis.

Generic name	Trade name and manufacturer	Dose	Dosing interval (h)	Route[a]	Indication
Hartman's solution	Lactated Ringer's, Travenol	As indicated	CRI[b]	IV[c]	Maintenance of water balance
Ampicillin	Penibrin, Teva Israel	25 mg/kg	8-h	IV	Antibacterial, broad spectrum
Metronidazol	Metronidazol, Braun, Germany	15 mg/kg	12-h	IV	Antibacterial, anaerobes
Ranitidine	Zantac, GSK	2 mg/kg	12-h	IV	Gastroprotectant, H2 receptor blocker
Metoclopramide	Pramin, Rafa	0.4 mg/kg	8-h	SC[d]	Antiemetic
Ondansetron	Zofran, GSK	0.15 mg/kg	12-h	IV	Antiemetic
Vitamin K	Konakion, Roche	2 mg/kg	24-h	SC	Hepatic support, coagulation factor synthesis
N-acetyl-cysteine	Flumil, Pharmazan	70 mg/kg	12-h	IV	Hepatic support, thiol group donor
SAMe[e]	SAME, Subherb	20 mg/kg	12-h	PO[f]	Hepatic support, thiol group donor
Lactulose	Lavolac, Perrigo	0.5 ml/kg	12-h	PO	Ammonia trapping
Vitamin E	Evitol, Teva	10 U/kg	24-h	PO	Hepatic support, lipid soluble antioxidant
Zinc-sulphate	Avazinc, Rekah	5 mg/kg	24-h	PO	Fat soluble Antioxidant
Mulivitamin	Duphafral, Duphar	1 ml/10 kg	24-h	SC	Hepatic support, multivitamin supplementation

[a] Route – route of administration.
[b] CRI – constant rate infusion.
[c] IV – intravenous.
[d] SC – subcutaneous.
[e] SAME – S-adenosyl-methionine.
[f] PO – orally.

compared to survivors (25/34 dogs; 74% *vs*. 4/16 dogs; 25%, $P = 0.002$; Table 1). Acute kidney injury (AKI) was diagnosed in 2 dogs (4%). Two non-survivors developed bacterial endocarditis and sepsis (one during hospitalization, and another, two weeks post-discharge). Based on presence of neurological abnormalities and plasma ammonia concentration, HE was present in 21/50 (35%) dogs and was significantly more frequent in non-survivors compared to survivors (58.8% *vs*. 6.3%, $P = 0.001$).

Overall mortality was 68% (34/50 dogs), 30 dogs diet naturally and 4 dogs were euthanased. Dogs were euthanased only when the prognosis was considered grave and to prevent suffering. Financial considerations did not play a role in the decision to perform euthanasia in any of the dogs. Survival rate was significantly ($P = 0.016$) higher in seven dogs that were presented with absence of clinical signs (5/7 dogs, 71%) compared to the rest of the cohort population (9/43 dogs; 26%).

3.9. Necropsy findings

Necropsy was performed in 15 dogs. Gross findings included enlarged yellow liver, icterus, diffuse haemorrhages, consistent with haemorraghic diathesis and DIC (15/15 dogs) abdominal effusion (12/15) and pleural effusion (3/15). Histopatological findings included characteristic aflatoxicosis-related finding, including hepatocellular degeneration, diffuse hepatocyte lipid vacuolation and fibrosis, bile ducts hyperplasia, fibrosis and multifocal haemorrhages (15/15 each). In addition, other findings included endocarditis (2/15) and acute tubular necrosis (1/15).

4. Discussion

Aflatoxicosis is an uncommon, infrequently reported intoxication in dogs (Bastianello et al., 1987; Newman et al., 2007; Dereszynski et al., 2008), with only a single, large-scale, retrospective study of a foodborne outbreak, described in the USA (Dereszynski et al., 2008). In contrast to this previous report (Dereszynski et al., 2008), dogs in the present study were all managed in a single institution, and thus, treatment and monitoring were similar. Monitoring included periodic full biochemistry panels, CBC, several haemostatic tests (i.e., PT. aPTT, fibrinogen and ATA) and plasma ammonia. These provided an opportunity to assess trends in clinical signs, laboratory tests and complication over time, and to perform a comprehensive risk factor analysis.

Aflatoxicosis is associated with non-specific clinical signs, and is thus mostly diagnosed post mortem, however, the present study, as the previous one of the USA outbreak (Dereszynski et al., 2008), includes dogs in which aflatoxicosis was diagnosed ante mortem. The source of the aflatoxin-contaminated canine diet responsible for the present outbreak is the same as the one that has led to the USA outbreak (Dereszynski et al., 2008; FDA-report, 2010). Because the Israeli distributer of the diet manufacturer covered the medical costs of all affected dogs, treatment, laboratory tests and hospitalisation were not limited by financial constraints. Therefore, affected dogs were immediately hospitalised in an intensive care unit, received symptomatic and supportive care and were constantly monitored. Treatment also included intensive FFP transfusion therapy. Thus, the present study provides extensive information of 50 dogs, obtained over a relatively long period, and is wider compared to previous reports of aflatoxicosis in dogs. In the study by Dereszynski et al. (2008) only 22 dogs were clinically followed in the hospital, and haemostatic tests were available in 25 dogs.

4.1. Signalment and clinical signs

Large breed dogs were presently mostly affected, while small-breed dogs were under-represented compared to the HUVTH population, most likely because the contaminated diet batch was a large-breed, adult diet. Due to the relatively long exposure time to the diet prior to presentation (45 days), this toxicosis should probably be regarded as subchronic, and a cumulative effect of the aflatoxin was likely. Thus, when clinical suspicion of aflatoxicosis arises, a detailed history should be obtained, with emphasis on the dog's diet over the previous one to eight weeks. This presently observed exposure-time is in agreement with previous experimental findings in dogs, in which a daily exposure to aflatoxins at 0.02 mg/kg/d led to clinical disease and typical hepatic histopathological lesions only after 10 weeks (Ketterer et al., 1975). Interestingly, presently, there was no difference between the consumption time-period of the contaminated diet and the body weight between the outcome groups, suggesting that in addition to the aflatoxin dose, individual susceptibility differences played a role in the outcome. Variations in daily consumption of the diet between dogs might have also influenced the outcome. Furthermore, it is possible that due to an uneven distribution of aflatoxin in the contaminated diet, non-survivor dogs were intermittently exposed to higher doses compared to surviving dogs. However, retrospectively, this hypothesis cannot be tested.

Most of the clinical signs observed presently were non-specific, as reported previously in studies of naturally-occurring and experi-

mental aflatoxicosis in dogs (Chaffee et al., 1969; Greene et al., 1977; Dereszynski et al., 2008). Peripheral edema, observed in 10% of the present dogs, was reported previously in 15/22 dogs with aflatoxicosis, and likely resulted from hypoalbuminaemia (Dereszynski et al., 2008). Icterus, neurological abnormalities and signs of bleeding (i.e., haematuria, haematochezia and melena) were significantly more common in non-survivors compared to survivors. The latter were reported previously in later stages of aflatoxicosis (Dereszynski et al., 2008). Neurological abnormalities, commonly observed in this study, as well as in a previous study (Dereszynski et al., 2008), were most likely due to HE, corresponding to the high recorded frequency of hyperammonaemia. Presence of such signs should probably be regarded as a negative prognostic signs, as these were significantly more common in non-survivors compared to survivors.

4.2. Laboratory abnormalities

Haemostatic abnormalities at presentation, including hypoantithrombinaemia, prolonged PT and aPTT and thrombocytopenia were very common in this study, as has been reported previously in 25 dogs with aflatoxicosis (Dereszynski et al., 2008). Decreased liver functions with subsequent failure very likely led to decreased production of coagulation factors and their inhibitors (i.e. antithrombin), leading to prolongation of clotting times and hypoantithrombinemia, and subsequently culminating in DIC (Prins et al., 2010). DIC in turn, probably worsened the thrombocytopenia. However, thrombocytopenia in liver diseases has also been associated with hypersplenism and portal hypertension, resulting in splenic congestion and platelet sequestration, as well as with decreased levels of thrombopoietin, produced by the liver (Peck-Radosavljevic, 2000). Platelet dysfunction might also occur in liver failure due to decreased fibrin/ogen degradation products clearance, which adhere to platelets and vascular endothelium (Peck-Radosavljevic, 2000). In addition to liver failure, it has been suggested that aflatoxicosis might induce DIC due to severe hepatic necrosis, leading to release of tissue thromboplastin-like substances from injured and necrotic hepatocytes (Greene et al., 1977; Bastianello et al., 1987; Scudamore et al., 1997; Leung et al., 2006; Newman et al., 2007; Dereszynski et al., 2008; Tang et al., 2009). Finally, it has been suggested that aflatoxin might act directly as anti-coagulant, due to its coumarin-like structure (Zimmerman, 1999).

Due to the presently documented severe haemostatic test abnormalities, all dogs (with exception of a single case) received constant FFP infusion (at 2 ml/kg/h) throughout hospitalisation, with no difference between survivors and non-survivors. Nevertheless, throughout hospitalisation, survivors had significantly shorter PT and aPTT compared to non-survivors. Although following initiation of FFP therapy, an initial gradual decline in PT and aPTT was observed in both outcome groups, non-survivors had a short-lived response despite continuous FFP therapy. Because DIC was present in most non-survivors, and significantly more so compared to survivors, rapid, severe consumption of coagulation factors and antithrombin with a more severe production failure were likely major contributors for this observed difference. It is thus not surprising that all three haemostatic analytes (e.g., PT, aPTT and ATA) were sensitive and specific predictors of the outcome, as has been previously reported (Dereszynski et al., 2008). In a previous study of aflatoxicosis in dogs, protein C activity, another haemostatic analyte, was measured in 25 dogs and was also significantly lower in non-survivors compared to survivors (Dereszynski et al., 2008), probably also reflecting presence of DIC in these dogs, and in agreement with the present observation.

In order to normalise coagulation parameters in non-survivors, a more aggressive treatment (i.e., higher FFP doses) was probably warranted, although the severe coagulation derangement might not have been resolved in the non-survivors due to the severe, irreversible liver failure. Presence of a more severe liver injury in the non-survivors is also supported by higher hepatocellular enzymes activity in this group. Although leakage of hepatocelluar enzymes does not reflect decreased liver functions, presently, there were significant correlations between AST activity and certain liver functions (e.g., serum bilirubin concentration) and haemostatic analytes (i.e., PT, aPTT and ATA). Additionally, serum bilirubin and cholesterol concentrations at presentation, both of which can be regarded as liver function tests, were higher in non-survivors, indicating presence of a more severe liver dysfunction in this group, in addition to marked hepatic injury, reflected by increased liver enzymes activity. The aforementioned evidence suggests that in dogs with aflatoxicosis the severity of liver injury and dysfunction is associated with the severity of haemostatic derangement and depletion of procoagulants and anticoagulants. Based on the present results, haemostatic tests (e.g., PT, aPTT and ATA) can be used as markers of the severity of intoxication, as well as prognostic indicators, in canine aflatoxicosis, because these were more severely deranged at presentation, as well as throughout hospitalisation, in non-survivors. Normalisation of these tests can likely be an indication of a successful treatment and a positive outcome.

Both outcome groups had liver damage, reflected by increased hepatobiliary enzymes activity. When comparing the activities of hepatobilliary enzymes between outcome groups, only ALP and AST, but not ALT, were significantly higher in non-survivors compared to survivors, which is in partial agreement with previous findings (Dereszynski et al., 2008). Because in most dogs CK activity was within RI, and there was no correlation between its activity and ALT or AST activities, the increase of the latter must be attributed to liver damage. Although serum hepatocellular enzymes activity is an indicator of hepatocyte injury, it has been reported previously in experimental and clinical aflatoxicosis in dogs that the magnitude of increase in hepatobilliary enzymes activity does not always correlate with the severity of the hepatocellular damage as observed at necropsy (Dereszynski et al., 2008). Possibly, since ALT is a cytosolic enzyme (Hoffmann and Solter, 2008), and leaks from injured hepatocytes both in milder as well as in more severe lesions, it was not a good discriminator between the outcome groups at presentation. In contrast, AST is present in both the cytoplasm and mitochondria of hepatocytes and leaks upon a more severe liver injury (Hoffmann and Solter, 2008), and thus, its serum activity was a better discriminator of the severity of the injury in aflatoxicosis. However, at later stages of aflatoxicosis, ALT activity probably did reflect the severity of hepatic injury, as its maximal activity during the disease course was significantly higher in non-survivors.

Presently, hypocholestrolaemia, hypoalbuminaemia and hyperbilirubinaemia were very common at presentation, as previously reported in dogs with aflatoxicosis (Dereszynski et al., 2008). However, at presentation, only a small proportion of the survivors showed hyperbilirubinaemia and hycholesterolaemia, and none showed hypoalbuminaemia. In contrast to a previous study of aflatoxicosis in dogs (Dereszynski et al., 2008), these analytes were not highly sensitive screening tests to identify subclinical aflatoxicosis. Because albumin, cholesterol and bilirubin concentrations are indicators of liver function, in contrast to hepatobilliary enzymes activity, their serum concentrations might not reflect early, milder liver injury. Nonetheless, these analytes can be used as markers of the severity of liver injury and failure as demonstrated presently by the ROC analyses. Bilirubin concentration was significantly higher, while cholesterol and albumin concentrations were significantly lower, in non-survivors compared to survivors throughout hospitalisation. One must consider that cholesterol concentration in aflatoxicosis is influenced by liver function but also by cholestasis, which can result from bile duct fibrosis, a commonly observed finding at necropsy.

4.3. Treatment

The treatment of aflatoxicosis is essentially supportive and symptomatic. Its goals should include aflatoxin elimination, prevention of further damage, hepatic protection and haemostaic stabilisation. Intravenous fluid therapy is essential in aflatoxicosis for correction of dehydration and hypovolaemia, but also to facilitate renal elimination of the reactive aflatoxin metabolites (M1, P1 and Q1) (Bingham et al., 2004). Vitamin E was administered as a lipid soluble antioxidant. In a recent study in people there was a significant strong negative correlation between serum AFB1 and vitamin E levels (Tang et al., 2009). Vitamin K was supplemented for its role in coagulation factors production, and because AFB1 might possess a coumarin-like anticoagulant effect (Zimmerman, 1999). Intravenous FFP was administered to provide procoagulant and anticoagulant proteins, as well as albumin, all of which were consumed, or lost through bleeding, and due to liver production failure.

Despite the present intensive treatment, the mortality rate was 68%, which is similar to previous findings in foodborne aflatoxicosis in dogs (64%) (Dereszynski et al., 2008). However, the mortality rate was much lower in the seven dogs that were presented with absence of clinical signs (24%), although they did present with laboratory abnormalities of liver injury and haemostatic abnormalities (i.e., increased ALP, ALT and AST activities, thrombocytopenia, prolonged PT and aPTT and hypoantithrombinaemia), and although all developed clinical signs of aflatoxicosis later during hospitalisation. Thus, hepatobilliary enzymes activity might serve as early biomarkers of aflatoxicosis, and can be used for screening when the history supports an aflatoxin exposure. If abnormalities are detected in such cases, early medical intervention should be commenced. The more favorable outcome of these dogs suggests that an intensive treatment can potentially be effective. In a previous study of aflatoxicosis in dogs, those that were presented with laboratory abnormalities, but with absence of clinical signs, did not receive treatment. Some of these animals did develop clinical signs later on, and thus the authors have suggested that early medical intervention was probably indicated (Dereszynski et al., 2008).

In conclusion, dogs with aflatoxicosis suffer severe liver injury and failure, commonly resulting in HE and DIC. Increased liver enzymes activity and coagulation tests abnormalities might serve as early indicators of intoxication in suspected animals. Several laboratory analytes that might facilitate prognostic projections at presentation were identified. Increased PT and aPTT, hypoantithrombinaemia were significantly more common and more severe in non-survivors compared to survivors and likely result from both liver failure and DIC. Hyperbilirubinaemia, hpocholesterolaemia and hypoalbuminaemia were also more common and more severe in non-survivors compared to survivors, and likely reflected the severity of liver failure. The mortality rate in aflatoxicosis in dogs is high despite intensive therapy. Dogs that presented earlier in the disease course when clinical signs were absent, and received immediate treatment, had lower mortality, and thus early intervention is indicated in such cases.

5. Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper. All medical costs of the dogs included in this study were met by C.T.S, Tel-Aviv, Israel, the Israeli distributor of Diamond Pet Foods, USA. Thus, costs did not limit any diagnostic or therapeutic measure in any of the dogs included in this study.

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