Investigation of forage mycotoxin levels in horses with biochemical evidence of liver disease or injury

Annabelle Graham[1†], Catriona Mackenzie[1], Victoria Colgate[2], Emily Floyd[1]

[1]Rossdales Ltd, Newmarket, UK

[2]University of Cambridge, Department of Veterinary Medicine, Cambridge, UK

[†] Present address: University of Liverpool Equine Hospital, Neston, UK

Corresponding author (all correspondences) - Annabelle Graham: aegraham@liverpool.ac.uk,

Catriona Mackenzie: catriona.mackenzie@rossdales.com

Victoria Colgate: vac33@cam.ac.uk

Emily Floyd: emily.floyd@rossdales.com

Keywords: horse, mycotoxin, forage, liver enzymes, Alltech

Abstract

Background: Mycotoxins are released by moulds and are naturally occurring toxic metabolites in cereals and forage that contribute to disorders ranging from reduced productivity to death. Little is known about the exposure and impact of multiple mycotoxins in horses in the UK. Objectives: To identify the prevalence and concentrations of mycotoxins found in forage fed to horses in the UK with biochemical evidence of liver disease or injury. Study Design: Retrospective case series. Methods: Records of forage mycotoxin sampling undertaken for horses with biochemical evidence of liver disease or injury between May 2019-October 2021 were reviewed. The quantity and frequency of 54 mycotoxins identified were recorded. Mycotoxins were grouped based on their biochemical structure. Results: Mycotoxins were detected in 50/52 (96%, CI:87-99) of forage samples; 42/52 (81%,CI:67-90) had ≥ 2 groups present (median:3). Emerging mycotoxins detected in 39/52 (75%,CI:61-86) with median concentration of 92µg/kg [IQR:20-444] (median concentration [IQR]); fusaric acid in 25/52 (48%,CI:34-62), (14 [11-45]); type B trichothecenes in 24/52 (46%,CI:32-61), (119 [50-1517]). One or more mycotoxin groups were detected in 14/52 (27%,CI:16-42) at a 'higher' risk concentration to animal health; 22/52 (42%,CI:29-57) samples had ≥ 1 mycotoxins groups detected at 'medium' or 'higher' risk concentrations. Main limitations: Lack of control population and potential for case selection bias. Conclusions: Mycotoxins are frequently found in forage eaten by horses with biochemical evidence of liver disease or injury but no causation can be concluded from this study. The effects of mycotoxins in horses and synergistic effects of multiple mycotoxins in horses warrant further investigation.

1. Introduction

Mycotoxins are naturally occurring toxic metabolites released by moulds and fungi. They grow on a variety of feed and crops, most commonly in wet and humid conditions. Over 500 different mycotoxins have been discovered to date [1]. In animals, mycotoxins can contribute to respiratory, reproductive, immunological, gastrointestinal and other disorders resulting in signs ranging from reduced productivity to death [2]. However, not all mycotoxins cause serious acute disease and the effects of many are not well understood. In contrast to intensively farmed animals, little is known about the impact of mycotoxins in horses. Being a monogastric non-ruminant species, it has been hypothesised that horses may be more sensitive than ruminants towards adverse effects of mycotoxins [3]. Increased liver enzymes are reported in response to mycotoxicosis in horses, as in other species [4, 5].

Globally, the most widely detected mycotoxins in animal feed or forage are produced by fusarium species; the most commonly reported is deoxynivalenol [5,6]. However, to date there is only one study reporting on mycotoxin found in commercial horse feed [3]. They concluded that “co-contamination with several mycotoxins is very common in commercial horse feed” [3]. However, in most samples the toxin concentrations were well below the levels which are usually considered as critical or even toxic [3]. There are only two studies to date that has investigated mycotoxin levels in forage (hay or grass) intended for horses [2,4]. In the North American study, deoxynivalenol, T2 toxin and zearalenone were found in forage, with deoxynivalenol present in the highest amounts that could impact horse health [2]. Durham, (2022), found that fumonisin B1 may be associated with outbreaks of liver disease [4]. However, studies have also found mycotoxins in a high proportion of forage fed to the control groups [4,7]. Our understanding of what mycotoxins horses are exposed to in forage is limited and even less is published regarding which mycotoxins could be clinically significant in horses.

This retrospective study aimed to present the data collected from forage sampling undertaken on horses with biochemical evidence of liver disease or injury between May 2019 to October 2021. The primary aim was to identify if mycotoxins are identified in forage of horses that presented with biochemical evidence of liver disease or injury and which mycotoxins are commonly detected. Additionally, we aimed to investigate the forage mycotoxin concentrations of those detected. The information collected in this pilot study should

provide a foundation for further, more in-depth, research into the mycotoxins commonly found in equine forage in the UK and their potential for causing disease.

2. Materials and methods

Electronic patient records were manually searched to retrospectively collect data from client submission forms submitted with forage samples to Rossdales Laboratories prior to mycotoxin testing. Data collected:

- Age, sex, breed
- Geographical location (postcode) of pasture/forage sampling
- Supplementary feeding, including if a mycotoxin binder has been used
- Sample type: grass, hay or haylage
- Clinical signs/ reason for testing
- If liver enzyme concentrations (GGT, GLDH, SAP, AST) or bile acid concentration had been detected outside of the laboratory reference ranges.

Forage samples were taken by clients and submitted to Rossdales Laboratories. All clients were advised to sample the centre of multiple different hay bales (five to six). For grass sampling, clients were advised to take five small handfuls from across the whole pasture and not to include soil. Clients were advised to post the samples early in the week (Monday or Tuesday) where possible, to avoid any delays in processing over the weekend. To be included in the study, horses must have had liver enzyme concentrations (GGT, GLDH, AST, SAP) or bile acid concentration had been detected outside of the laboratory reference range on a blood sample (confirmed by Rossdales Laboratories or the referring veterinary surgeon) and the forage sample must be submitted with a completed client submission form.

Samples were sent to Alltech and tested for percentage dry matter and then tested for 54 mycotoxins (see appendix one for list of mycotoxins tested) using liquid chromatography and mass spectrometry techniques [8]. Samples were ground in a coffee grinder for 30 seconds to obtain consistent particle size. 400mg sub-samples were taken and equally distributed in glass reaction vials. The samples were centrifuged at 4000rpm for 30 minutes. 0.5mL of supernatant was collected and dried under a nitrogen stream for 30 minutes at room temperature. The samples were reconstituted in 0.5mL of loading buffer. The analysis was

performed on Acquity UPLC/ESI-TQD MS/MS system utilising an ethylene-bridged hybrid C18 analytical column maintained at 40 degrees centigrade. The analysis was carried out at a flow rate of 0.42ml/minute over 16 minutes per samples injection with a gradient of water. 54 mycotoxins were analysed and the detection limits, lower quantification limits and standard deviations were set by Alltech for each mycotoxin.

3. Analysis

Descriptive statistics were carried out for categorical data and summary statistics for quantitative data. If normally distributed (as determined by Shapiro Wilk Normality Test) means and confidence intervals were presented for quantitative data. If the data were not normally distributed medians and interquartile ranges were presented. The frequency of each mycotoxin detected was recorded to establish the most commonly detected mycotoxins and the median levels detected of those identified. Any detected values were reported as µg/kg dry matter. Adverse performance risks associated with multiple mycotoxins in feed were evaluated by calculating a risk equivalent quantity (REQ) [9] . REQ represents the sum of the mycotoxin risk based on the mycotoxin concentration and respective risk factor [9]. A species-specific risk equivalence factor is assigned to each mycotoxin relative to the most toxic mycotoxin (aflatoxin B1) [9]. The total toxicity of multiple mycotoxins can then be hypothesised as a single risk equivalent quantity (REQ), which is calculated by summing the products of individual REFs and their respective concentrations [9].

4. Results

A total of 78 forage samples were submitted to Rossdales Laboratories for testing by Alltech between May 2019 and October 2021. Of those tested, 52 samples fulfilled the selection criteria (see appendix 2). 27 samples of grass (52%), one sample of haylage (2%) and 24 samples of hay (46%) were submitted from 46 cases (six horses were submitted with two or more forage samples). Ages of horses ranged from 2-32 years old, with age unspecified in 8 horses (median age 12 years old, with an interquartile range of 6.75-19 years). The predominant breed was cobs (n=10), with mixed representation from other breeds (pony = 8, warmblood = 7, miniature = 3, thoroughbred = 3, Irish sport horse = 2, arabian = 1, hackney = 1, suffolk = 1, unspecified = 10). All horses had increased liver enzyme concentrations (GGT, GLDH, AST, SAP) with or without bile acid concentration detected outside of the laboratory reference range on blood serum

analysis by either Rossdales Laboratories or by the referring veterinary surgeon with a median 28 days (IQR 21-60 days) of liver enzyme analysis prior to mycotoxin forage analysis. To be included, all cases had confirmed increases in liver enzymes however, only 18/52 samples had some or all data available or detailed on the client submission form for liver enzyme and bile acid values (see table one).

Geographical distribution was predominant focused in the southeast of England with all but one sample (300 miles) within 120 miles of Newmarket, UK. Mycotoxins were detected in 50/52 samples. Two or more groups were detected in 42/52 samples, with the highest number of six mycotoxins groups detected (n=1). Toxins were detected from all groups except aflatoxins. The median number of mycotoxin groups detected in each sample was three (see figure one). The most commonly detected groups were emerging mycotoxins (n=39), fusaric acid (n=25), followed by type b trichothecenes (n=24) (see table two and figure two).

Based on current research and published data for other species, Alltech quantify individual mycotoxins risk to the animal as lower, medium or higher risk. All individual mycotoxins groups identified were detected at median concentration levels of 'lower' or below except Ochratoxins/citrinin (AB,B) which were 'higher' with a median concentration of 66 µg/kg [IQR 22-66 µg/kg] (see table three). Type B trichothecenes were most commonly identified at medium or high-risk concentrations (8/24 samples) (see figure three). 14/52 (27%) samples had one or more mycotoxin group that was detected at the concentration above the 'higher' risk threshold, 22/52 (42%) samples had one or more mycotoxins groups that were detected at concentrations at 'medium' or 'higher' risk.

5. Limitations

The major limitation to the study was case selection bias and a lack of a control group. This was impossible to mitigate due to the method of data collection and retrospective nature of the study. Incomplete data sets were also a problem and was the most common reason for samples not meeting inclusion criteria. Data quantifying the degree of increase in liver enzyme and bile acid concentrations were only available in 18/52 samples. The growth of mycotoxins is affected by multiple factors such as environmental conditions such as temperature, moisture conditions, geography and agricultural practices. As these factors vary both

seasonally and annually, levels of mycotoxins will also vary. Due to the short study duration and low sample numbers, it was not possible to investigate this further. There was no data available for mycotoxins in hard or concentrate feed, which may have also been a source of mycotoxins for horses fed concentrates in addition to forage. Due to the retrospective nature of the study, it was impossible to control the method and timing of the forage sampling. All clients were given the same advice for sampling, but the timing of mycotoxin testing after increased liver enzyme detection could not be controlled. Due to the lack of evidence regarding mycotoxins and their effects in horses, reference ranges were extrapolated from food animals. Alltech evaluate the impact (lower/moderate/higher) of mycotoxins concentrations detected where an impact on performance and health has been observed at chronic levels of exposure in farm animals, rather than toxicological limits. No such data is available in horses. Alltech set the reference limits based on a variety of sources including research and government regulations, with support from commercial observations. There is very little data to demonstrate effects of mycotoxins on horses. Due to the lack of data in horses that indicate which mycotoxins are prevalent or clinically significant, the 54 mycotoxins tested were selected because they most commonly affect food animal health and productivity.

6. Discussion

There is very little published data investigating mycotoxin level in forage in the UK. It is well documented that the source of mycotoxin feed contamination is more likely to originate from processed grains or feed than grass or hay that undergoes comparatively less storage or processing [10]. Mycotoxins are often not homogenously dispersed in the feed and this problem is even more apparent when sampling grass across a field [11]. Mycotoxins may therefore stay analytically undetected, even with optimal sampling procedures [12]. However, the risk of mycotoxin-contaminated forage has been documented [2,4], and confirmed in this study, where mycotoxins were identified in 96% of forage samples.

This paper made no attempt to draw causation between mycotoxin ingestion and biochemical evidence of liver disease or injury. There are many and complex reasons for increased liver enzyme or bile acid concentrations including ingestion of mycotoxins [4,5]. We included samples from horses with biochemical

evidence of liver disease or injury to identify mycotoxins to guide further research, not to draw associations between the mycotoxin exposure and liver disease where a control group would be necessary.

The most commonly detected mycotoxin group was emerging mycotoxins, found in 75% (39/52) of samples. However, when identified, emerging mycotoxins were found at concentrations that are not considered a risk to equine health. This contrasts with type B trichothecenes, which although identified in 46% (24/52) samples, was more commonly found at significant concentrations. In 8/24 samples type B trichothecenes were identified at medium or high-risk concentrations. Type B trichothecenes are produced by fusarium moulds and are frequently identified in forage in Europe [13]. They can cause significant gastrointestinal disease in humans and pigs from both acute and chronic exposure [14]. Feed refusal and gastrointestinal erosions have been noted in pigs after chronic exposure to deoxynivalenol (DON), which is a type of Type B trichothecenes [14]. DON was found more commonly in colic cases compared to the control group in one study [7]. In a study by Raymon et. al in 2003, the impact of fusarium mycotoxins fed to horses (DON (14,000 ug/kg), fusaric acid (6400 mg/kg) and zearalenone (2000 ug/kg)) was demonstrated by a significant reduction in feed consumption and GGT significantly increased compared to control day 7-14 [5]. They concluded that exercised horses are also susceptible to fusarium mycotoxicosis as indicated by appetite suppression and weight loss when feeding contaminated feed with fusarium mycotoxins for 21 days [15]. Whilst these studies demonstrated clinical effects of significant fusarium exposure in horses, no histology was performed, and study duration was limited to 21 days. More research is needed to establish subclinical effects as well as the effects of longer exposure and lower doses.

The lack of a control group was a significant limitation of the study. Previous studies identifying mycotoxin exposure of horses with colic and liver disease, also identified mycotoxins in control populations [4,7]. Whilst we cannot conclude in this study if the biochemical evidence of liver disease or injury were related to the mycotoxin exposure, it has demonstrated the frequency at which mycotoxins are identified in UK forage. Despite being found in control populations in other studies, there is insufficient data to conclude that mycotoxins are not potentially significant to equine health. Exposure to high levels has been demonstrated

to cause acute disease, but no long-term cohort studies have been performed in horses to assess long term consequences [5,15] .

No studies have quantified the cumulative risk of multiple mycotoxins on horse health. Moulds can produce multiple mycotoxins and there is evidence of the synergistic effects of fusarium mycotoxins [16]. Adverse performance risks associated with multiple mycotoxin in feed can be evaluated in farm animals to calculate a risk equivalent quantity (REQ) [9]. In this study, 40/52 samples had two or more groups of mycotoxins detected and 25/52 samples had a medium or greater REQ. This suggests that the number of mycotoxins identified should be considered in addition to the type and concentration of mycotoxin detected. However, further work is needed to establish both the effects of individual and multiple mycotoxins on horses.

7. Conclusion

Whilst the study data cannot be used to draw causation between mycotoxins and liver disease, it has shown that multiple mycotoxins are frequently found in the forage eaten by horses with biochemical evidence of liver disease or injury. Emerging mycotoxins were most commonly identified, type B trichothecenes were most commonly detected at levels that could be a risk to equine health. Nearly half of samples had one or more mycotoxins groups that were detected at a concentration that was ‘medium’ or ‘higher’ risk to animal health. The effects of mycotoxins in horses and synergistic effects of multiple mycotoxins in horses warrant further investigation.

Declarations

No conflicts of interest have been declared.

Acknowledgements

The authors would like to thank Alltech for providing supporting materials and information on their mycotoxin research.

Sources of Funding

No financial support was obtained from any organisation for the work performed in this study.

Ethical animal research

No ethics review was necessary for this retrospective study.

References

[1] Alshannaq, A. & Yu, J.-H. Occurrence, Toxicity, and Analysis of Major Mycotoxins in Food. International Journal Environmental Research and Public Health 2017; 14(6), 632. doi: 10.3390/ijerph14060632

[2] Raymond, S. An investigation of the concentratios of selected fusarium mycotoxins and the degree of mold contamination of Ontario field-dried hay. Journal Equine Veterinary Science 2000; 20(10), 616-621. doi: 10.1016/S0737-0806(00)80403-7

[3] Liesener, K; Usleber, E; Curtui, V; Dietrich, R; Martlbauer, E. Mycotoxins in horse feed. Mycotoxin Research 2009; 26(1), 23-30. DOI: 10.1007/s12550-009-0037-8

[4] Durham, A. Association between forage mycotoxins and liver disease in horses. Journal of Veterinary Internal Medicine 2022;. 36(4), 1502–1507. doi:10.1111/jvim.16486

[5] Raymond, S. L; Smith, T. K; Swamy, H. L. Effects of feeding a blend of grains naturally contaminated with Fusarium mycotoxins on feed intake, serum chemistry, and haematology of horses, and the efficacy of a polymeric glucomannan mycotoxin adsorbent. *Journal of anim*al science 2003; 81(9), 2123-2130. doi: 10.2527/2003.8192123x

[6] Smith, T. K., McMillan, E. G. & Castillo, J. B. Effect of feeding blends of Fusarium mycotoxin-contaminated grains containing deoxynivalenol and fusaric acid on growth and feed consumption of immature swine. Journal of Animal Science 1997; 75(8), 2184-2191. doi: 10.2527/1997.7582184x.

[7] Dänicke, S; Saltzmann, J; Liermann, W; Glatter, M; Hüther, L; Kersten, S; Zeyner, A; Feige, K; Warnken, T. Evaluation of Inner Exposure of Horses to Zearalenone (ZEN), Deoxynivalenol (DON) and Their Metabolites in Relation to Colic and Health-Related Clinical-Chemical Traits. Toxins 2021; 13(8), 588. doi: 10.3390/toxins13080588

[8] Jackson, L. C; Kudupoje, M. B; Yiannikouris, A. Simultaneous multiple mycotoxin quantification in feed samples using three isotopically labeled internal standards applied for isotopic dilution and data normalization through ultra-performance liquid chromatography/electrospray ionization tandem MS. Rapid communications in mass spectrometry 2012; 26(23), 2697-2713. doi: 10.1002/rcm.6405

[9] Yiannikouris, A. Risk Equivalence factor: a novel approach for estimating performance impact of multitoxin contamination. Mycosorb 29th Symposium 2013; poster 497

[10] Krizova, L; Dadakova, K; Dvorackova, M; Kasparovsky, T. Feedborne mycotoxins beauvericin and enniatins and livestock animals. Toxins 2012; 13(32), 1-14. doi: 10.3390/toxins13010032

[11] Skladanka, J; Adam, V; Dolezal, P; Nedelnik, J; Kizek, R; Linduskova, H; Mejia, J E A; Nawrath, A. How do grass species, season and ensiling influence mycotoxin content in forage? International Journal of Environmental Research and Public Health 2013; 10(11), 6084-6095. doi: 10.3390/ijerph10116084

[12] Binder, E. Managing the risk of mycotoxins in modern feed production. Animal Feed Science and Technology 2007; 133(1–2), 149-166. doi:10.1016/j.anifeedsci.2006.08.008

[13] Pereira, CS; Cunha, SC; Fernandes, JO. Prevalent mycotoxins in animal feed: occurrence and analytical methods. Toxins 2009; 11(5), 209. doi: 10.3390/toxins11050290

[14] Pinton, P. & Oswald, I. P. Effect of Deoxynivalenol and Other Type B Trichothecenes on the Intestine: A Review. Toxins 2014;. 6(5),1615-1643. doi: 10.3390/toxins6051615

[15] Raymond, S. L; Smith, T. K; Swamy, H. L. Effects of feeding a blend of grains naturally contaminated with Fusarium mycotoxins on feed intake, metabolism, and indices of athletic performance of exercised horses. Journal of Animal Science 2005; 83(6), 1267-1273. doi: 10.2527/2005.8361267x

[16] Alassane-Kpembi, I; Schatzmayr, G; Taranu, I; Marin, D; Puel, O; Oswald, I P. Mycotoxins co-contamination: Methodological aspects and biological relevance of combined toxicity studies. Critical Reviews in Food Science and Nutrition 2017; 57(16), 3489-3507. doi: 10.1080/10408398.2016.1140632.

2.3 Figures and appendices

Appendix 1 - List of the mycotoxins tested

Appendix 2 - case selection criteria

284

285

286

287 Appendix 1 - List of the mycotoxins tested

Group of mycotoxins	Mycotoxins tested							
Alflatoxin	B1, B2, G1, G2							
Ochratoxin/ Citrinin	Ochratoxin A, B/ Citrinin							
type B trichothecenes	3-AcDon, 15-AcDon	Deoxyivalenol	Fusarenon X	Nivalenol	DON-3-glucoside			
type A trichothecenes	T2 toxin	HT2 toxin	Neosolaniol	Diacetoxyscirpenol				
Fusaric acid	Fusaric acid							
emerging mycotoxins	Enniatin A, A1, B, B1	Alternaroil	Citreoviridin	Beauvericin	Moniliformin	Phomopsin A		
Ergot toxins	Ergometrin(in)e	Ergotamin(in)e	Ergocristin(in)e	Ergosin(in)e	Ergocornin(in)e	Ergocryptin(in)e	Methylerg-onovine	Lysergol
Fumonisins	Fumonisin B1, B2, B3							
Zearalenones	Zearalenone							

Penicillum toxins	Roquefortine C	Patulin	Cyclopiazonic acid	Wortmannin	Penicillic acid	Mycophenolic acid
Aspergillus toxins	Sterigmatocystin	Gliotoxin	Verruculogen			

288

289 Table 1 - biochemical analysis of liver disease or injury

290

Variable	Number of Cases	Cases without data	Minimum	Q1	Median	Q3	Maximum
GGT (iu/L)	17	35	64	125	202	993	1576
AST (iu/L)	15	37	451	598	723	94	1037
SAP (iu/L)	14	38	180	193	226	707	2703
BA (µmol/L)	17	35	2.4	6.6	10.8	26.0	92.0
GLDH (iu/L)	6	46	6	37	113	316	824
Days between liver and forage (days)	16	36	10	21	28	60	105

291

292 Table 2 – Groups of mycotoxins detected

Mycotoxin group	Number of cases	Minimum	Q1	Median	Q3	Maximum
		µg/kg				
Ochratoxins/Citrinin(AB, B)	3	22.0	22.0	66.0	66.0	66.0
Type B trichothecenes	24	20	50	119	1517	22907
Fusaric acid	25	9.0	11.0	14.0	45.0	755.0
Type A trichothecenes	4	15.0	22.5	67.0	182.0	213.0
Emerging	39	2	20	92	444	7032
Ergot toxins	8	1	6	22	87	8584
Fumonisins	14	17.0	23.5	51.0	143.3	462.0
Zearalenones	2	189.0	*	242.5	*	296.0
Penicillium toxins	6	6.0	13.5	29.5	85.0	97.0
Aspergillus toxins	13	3.0	8.5	17.0	73.5	225.0

293

294 Table 3 - Mycotoxins detected concentration and reference ranges from Alltech

295

Mycotoxin	Number of cases	Mycotoxin concentration (µg/kg)						
		Mean	SD	Median	IQR	Lower	Medium	Higher
Ochratoxins/citrinin (AB,B)	3	51	±25	66	22-66	20	35	50
Type B trichothecenes	24	1823	±4675	119	50-1517	500	1000	2000
Fusaric acid	25	85	±179	14	11-45	1000	2000	3000
Type A trichothecenes	4	91	±87	67	23-182	50	100	200
Emerging	39	495	±1190	92	20-444	500	1000	2000
Ergot toxins	8	1098	±3025	22	6-87	50	100	200
Fumonisins	14	100	±124	51	24-143	500	1000	1500
Zearalenones	2	243	±76	243	-	100	250	500
Penicillum toxins	6	43	±37	30	14-85	50	100	200
Aspergillus toxins	13	47	±64	17	9-74	50	100	200