Investigation of forage mycotoxin levels in horses with increased liver enzyme concentrations

Annabelle Bates[1], Catriona Mackenzie[2], Victoria Colgate[3], and Emily Floyd[2]

[1]Rossdales Equine Hospital and Diagnostic Centre
[2]Rossdale Equine Hospital
[3]University of Cambridge

December 2, 2022

Abstract

Background: Mycotoxins are naturally occurring toxic metabolites found in cereals and forage released by moulds and other fungi. In domestic farm animals, mycotoxins contribute to a spectrum of disorders. However, little is known about the impact of multiple mycotoxins in horses and there is little published data investigating mycotoxins found in forage fed to horses in the UK. Objectives: To identify the concentrations of mycotoxins found in forage fed to horses in the UK with increased liver enzyme concentrations. Study Design: Retrospective case series. Methods: Records of forage mycotoxin sampling undertaken for horses with increased liver enzymes between May 2019 – October 2021 were reviewed. The quantity and frequency of 54 mycotoxins identified were recorded. Individual mycotoxins were grouped based on their biochemical structure or fungus they are produced by. Results: Mycotoxins were detected in 50/52 (96%, CI 87-99) of forage samples; 42/52 (81%, CI 67-90) samples had two or more present. The median number of mycotoxin groups detected was three. Emerging mycotoxins were detected in 39/52 samples (75%, CI 61-86) with median concentration of 92 µg/kg [IQR 20-444] (median concentration [IQR]); fusaric acid in 25/52 samples (48%, CI 34-62), (14 [11-45]), and type B trichothecenes in 24/52 samples (46%, CI 32-61), (119 [50-1517]). One or more mycotoxin groups were detected in 14/52 samples (26%, CI 16-42) at a concentration thought to be 'higher' risk to animal health; 22/52 (42%, CI 29-57) samples had one or more mycotoxins groups that were detected at a concentration that was 'medium' or 'higher' risk. Main limitations: Lack of a control population and potential for case selection bias. Conclusions: Mycotoxins are frequently found in the forage eaten by horses with increased liver enzymes. The effects of mycotoxins in horses and synergistic effects of multiple mycotoxins in horses warrant further investigation.

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 (Alshannaq and Yu 2017). In animals, mycotoxins can contribute to respiratory, reproductive, immunological, gastrointestinal and other disorders resulting in signs ranging from reduced productivity to death (Raymond 2000). 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 (Liesener et al. 2009**).**Increased liver enzymes are reported in response to mycotoxicosis in horses, as in other species (Raymond et al. 2003; Durham 2022).

Globally, the most widely detected mycotoxins in animal feed or forage are produced by fusarium species; the most commonly reported is deoxynivalenol (Smith et al 1997; Raymond et al. 2003). However, to date there is only one study reporting on mycotoxin found in commercial horse feed (Liesener et al. 2009).

They concluded that "co-contamination with several mycotoxins is very common in commercial horse feed" (Liesener et al. 2009). However, in most samples the toxin concentrations were well below the levels which are usually considered as critical or even toxic (Liesener et al. 2009). There are only two studies to date that has investigated mycotoxin levels in forage (hay or grass) intended for horses (Raymond 2000, Durham 2022). In the North American study, they found deoxynivalenol, T2 toxin and zearalenone in forage, with deoxynivalenol present in the highest amounts that could impact horse health (Raymond 2000). Durham, 2022, found that fumonisin B1 may be associated with outbreaks of liver disease (Durham 2022). However, studies have also found mycotoxins in a high proportion of forage fed to the control groups (Dänicke et al. 2021; Durham 2022). 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 increased liver enzymes between May 2019 to October 2021. The primary aim was to identify if mycotoxins are identified in forage of horses that presented with increased liver enzymes 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.

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 increased liver enzyme concentrations had been detected

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 small handfuls from across the whole pasture. To be included in the study, horses must have had clinical signs of liver disease and/or increased liver enzyme concentrations on a blood sample (confirmed by Rossdales Laboratories or the referring veterinary surgeon) within 120 days of forage sampling; and must have been greater or equal to two years of age at the time of sample collection.

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 (Jackson et al. 2012). 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. 500uL of supernatant was collected and dried under a nitrogen stream for 30 minutes at room temperature. The samples were reconstitute in 500uL 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 degree centigrade. The analysis was carried out at a flow rate of 0.42ml/min over 16 minutes per samples injection with a gradient of water. 54 mycotoxins were anaylsed and the detection limits, lower quantification limits and standard deviations were set by Alltech for each mycotoxin.

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 was 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. Adverse performance risks associated with multiple mycotoxins in feed were evaluated by calculating a risk equivalent quantity (REQ) (Yiannikouris 2013). REQ represents the sum of the mycotoxin risk based on the mycotoxin concentration and respective risk factor (Yiannikouris 2013). A species-specific risk equivalence factor (REF) is assigned to each mycotoxin relative to the most toxic mycotoxin (aflatoxin B1). 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 (Yiannikouris 2013).

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 confirmed on blood serum analysis by either Rossdales Laboratories or by the referring veterinary surgeon with a median 28 days (IQR 21-60 days) between liver enzyme analysis and mycotoxin forage analysis. 18/52 samples had some or all data available for liver enzymes 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 was most commonly found at significant concentrations, with 8/24 samples type B trichothecenes identified at medium or high-risk concentrations (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.

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 demonstrating degree of increase in liver enzyme concentrations was 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. The spectrum mycotoxins tested are those the most commonly affect food animals which were selected to be based due to a lack of data in horses that indicate which mycotoxins are prevalent or clinically significant.

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 little or no storage or processing (Krizova et al. 2021). Mycotoxins are often not homogenously dispersed in the feed and this problem is even more apparent when sampling grass across a field (Skladanka et al. 2013). Mycotoxins may therefore stay analytically undetected, even with perfect sampling procedures (Binder 2007). However, the risk of mycotoxin-contaminated forage has been documented (Raymond 2000; Durham 2022), 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 increased liver enzyme concentrations. There are many and complex reasons for increased liver enzyme concentrations including ingestion of mycotoxins (Raymond et al. 2003; Durham 2022). We included samples from horses with increased liver enzyme concentrations to identify mycotoxins that may have some clinical relevance for horses and 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 (Pereira et al. 2019). They can cause significant gastrointestinal disease in humans and pigs from both acute and chronic exposure (Pinton and Oswald, 2014). Feed refusal and gastrointestinal erosions have been noted in pigs after chronic exposure to deoxynivalenol (DON), which is a type of Type B trichothecenes (Pinton and Oswald, 2014). DON was found more commonly in colic cases compared to the control group in one study (Dänicke et al. 2021). 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 (Raymond et al. 2003). 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 (Raymond et al. 2005). 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 (Dänicke et al. 2021; Durham 2022). Whilst we cannot conclude in this study if the increased liver enzyme concentrations 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 (Raymond et al. 2003; Raymond et al. 2005).

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 (Alassane-Kpembi et al. 2017). Adverse performance risks associated with multiple mycotoxin in feed can be evaluated in farm animals to calculate a risk equivalent quantity (REQ) (Yiannikouris, 2013). 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.

Conclusion

Multiple mycotoxins are frequently found in the forage eaten by horses with increased liver enzymes. 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

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

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

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

Dänicke, S; Saltzmann, J; Liermann, W; Glatter, M; Hüther, L; Kersten, S; Zeyner, A; Feige, K; Warnken, T (2021). 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.* 13(8), 588. doi: *10.3390/toxins13080588*

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

Jackson, L. C; Kudupoje, M. B; Yiannikouris, A (2012). 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.* 26(23), 2697-2713. doi: 10.1002/rcm.6405

Krizova, L; Dadakova, K; Dvorackova, M; Kasparovsky, T (2021). Feedborne mycotoxins beauvericin and enniatins and livestock animals.*Toxins.* 13(32), 1-14. doi: *10.3390/toxins13010032*

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

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

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

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

Raymond, S. L; Smith, T. K; Swamy, H. L (2003). 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 animal science.* 81(9), 2123-2130. doi: 10.2527/2003.8192123x

Raymond, S. L; Smith, T. K; Swamy, H. L (2005). 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* . 83(6), 1267-1273. doi: 10.2527/2005.8361267x

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

Smith, T. K., McMillan, E. G. & Castillo, J. B (1997). 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.* 75(8), 2184-2191. doi: 10.2527/1997.7582184x.

Yiannikouris, A (2013). Risk Equivalence factor: a novel approach for estimating performance impact of multitoxin contamination.*Mycosorb 29th Symposium* , poster 497

Figures and appendices

Appendix 1 - List of the mycotoxins tested

Group of mycotoxins	Mycotoxins tested	Mycotoxins tested	Mycotoxins tested	Mycotoxins
Alflatoxin	B1, B2, G1, G2	B1, B2, G1, G2	B1, B2, G1, G2	B1, B2, G1,
Ochratoxin/ Citrinin	Ochratoxin A, B/ Citrinin	Ochratoxin A, B/ Citrinin	Ochratoxin A, B/ Citrinin	Ochratoxin
type B trichothecenes	3-AcDon, 15-AcDon	Deoxyivalenol	Fusarenon X	Nivalenol
type A trichothecenes	T2 toxin	HT2 toxin	Neosolaniol	Diacetoxysc
Fusaric acid	Fusaric acid	Fusaric acid	Fusaric acid	Fusaric acid
emerging mycotoxins	Enniatin A, A1, B, B1	Alternaroil	Citreoviridin	Beauvericin

Group of mycotoxins	Mycotoxins tested	Mycotoxins tested	Mycotoxins tested	Mycotoxins
Ergot toxins	Ergometrin(in)e	Ergotamin(in)e	Ergocristin(in)e	Ergosin(in)e
Fumonisins	Fumonisin B1, B2, B3	Fumonisin B1, B2, B3	Fumonisin B1, B2, B3	Fumonisin B
Zearalenones	Zearalenone	Zearalenone	Zearalenone	Zearalenone
Penicillum toxins	Roquefortine C	Patulin	Cyclopiazonic acid	Wortmannin
Aspergillus toxins	Sterigmatocystin	Gliotoxin	Verruculogen	Verruculoge

Table 1 - liver enzyme analysis

Variable	Number of Cases	Cases without data	Minimum	Q1	Median	Q3	Maxim
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

Table 2 – Groups of mycotoxins detected

Mycotoxin group										
Mycotoxin group	Number of cases	Minimum	Q1	Median	Q3	Maximum	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	Ochratoxins/Citrin

Mycotoxin	Number of cases	Mycotoxin concentration (µg/kg)	Mycotoxin concentration (µg/kg)	M
		Mean	SD	M
Ochratoxins/citrinin (AB,B)	3	51	±25	6
Type B trichothecenes	24	1823	±4675	1
Fusaric acid	25	85	±179	1
Type A trichothecenes	4	91	±87	6
Emerging	39	495	±1190	9
Ergot toxins	8	1098	±3025	2
Fumonisins	14	100	±124	5
Zearalenones	2	243	±76	2
Penicillum toxins	6	43	±37	3
Aspergillus toxins	13	47	±64	1

Hosted file

`Figure 1.docx` available at https://authorea.com/users/562011/articles/609326-investigation-of-forage-mycotoxin-levels-in-horses-with-increased-liver-enzyme-concentrations

Hosted file

`Figure 2.docx` available at https://authorea.com/users/562011/articles/609326-investigation-of-forage-mycotoxin-levels-in-horses-with-increased-liver-enzyme-concentrations

Hosted file

Figure 3.docx available at https://authorea.com/users/562011/articles/609326-investigation-of-forage-mycotoxin-levels-in-horses-with-increased-liver-enzyme-concentrations

Hosted file

Appendix 2.docx available at https://authorea.com/users/562011/articles/609326-investigation-of-forage-mycotoxin-levels-in-horses-with-increased-liver-enzyme-concentrations