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Detection of gliotoxin in fungal culture supernatants and in urine and faecal samples of various domestic mammals

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INTRODUCTION

The structure of the mycotoxin gliotoxin (GT) is based on the metabolite epipolythiodioxopiperazine (ETP). The intact disulphide bridge within this cyclic dipeptide is of particular importance for its activity. This activity results in various effects, including cytotoxicity, genotoxicity, immunosuppression, apoptosis and others. GT production was first shown in Gliocladium fimbriatum. Other known GT producing species are *Trichoderma sp., Penicillium sp. and Aspergillus sp.* GT is considered an important virulence factor of *Aspergillus fumigatus* in clinical aspergillosis. The production of this mycotoxin is presumably influenced by stimuli such as pH-value and temperature as well as the N/C-availability where an increase of production was observed in times of deficiancy. Selected fungal culture supernatants as well as faecal and urine samples from various domestic mammals were examined for GT using a competitive ELISA (enzyme-linked immunosorbent assay). The examinations resulted in the mycotoxin being detected *in vivo*.

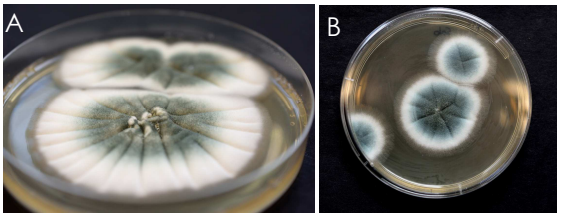
COLLECTION AND PREPARATION OF SAMPLES

Preparation of 25 different fungal culture supernatants:

- Initial cultivation on Sabouraud agar (28°C for 14 days)
- Inoculation of cell culture flasks with harvested cultures (Sabouraud or Czapek liquid medium)
- Transfer of 1 ml supernatant into Eppendorf tubes after 8 days; vortexing and centrifugation afterwards
- *Aspergillus sp., Penicillium sp., Candida sp., Geotrichum sp., Fusarium sp., Lichtheimia corymbifera, Paecilomyces lilacinus, Cheatominum globosum, Trichosporum asahiii*

Preparation of faecal and urine samples:

- Rectally collected faecal samples of horses and cattle
- Weighing and mixing with 1 ml PBS and 0.01% Tween; followed by several rounds of centrifugation
- Centrifugation of urine samples collected during micturition



Figures A, B
Aspergillus fumigatus on Sabouraud agar.

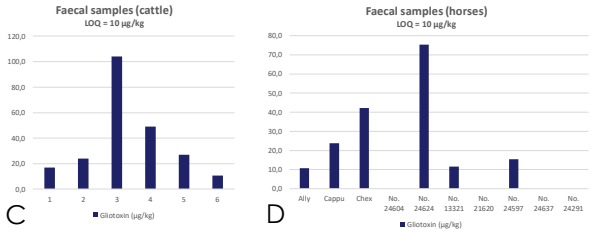
METHODOLOGY

Steps	Competitive ELISA for Gliotoxin detection
Coating with catcher antibodies	Anti-IgG(Fc)-rabbit-antibodies (extracted from goats): binding the anti-GT-antibody with its Fc region 0,1 M $NaHCO_3$ buffer solution (concentration: 2 µg/ml) 100 µl/well Incubation overnight (4°C)
Washing	2x with 0,9% NaCl, 0,05 % Tween 20
Adding the sample/ gliotoxin standard	Sample preparation: 90 µl BSA (bovine serum albumin) + 10 µl sample (supernatant) → 1:10-dilution Standard: bmtGliotoxin in PBS (0,1 % Tween 20, 0,05 % BSA) PBS: phosphate-buffered saline Concentration: 2500 ng/ml → 1:5-titration 50 µl/well
Adding the anti-GT-antibodies	IgG-anti-gliotoxin-antibodies (extracted from rabbit) in PBS, 0,1 % Tween 20, 0,05 % BSA, Concentration: 50 ng/ml 50 µl/well Put on platform shaker at room temperature for 1h (no washing afterwards!)
Adding the GT-HRP conjugate	Gliotoxin-bLA-HRP in PBS, 0,1 % Tween 20, 1 % BSA, bLA: bovine lactalbumin, HRP: horseradish peroxidase Concentration: 10 ng/ml 50 µl/well Put on platform shaker at room temperature for 1h
Washing	3x with 0,9% NaCl, 0,05 % Tween 20
Starting the colour reaction	Adding H_2O_2 and TMB (3/1 mM) + 0,2 M citrate buffer, pH 4,0 TMB: tetramethylbenzidine 100 µl/well Incubation at room temperature for 15 min
Adding the stop solution	1M H_2SO_4 50 µl/well
Photometric measurement	at 450 nm (Ref.: 620 nm)

RESULTS

	Sabouraud ng/ml	Czapek ng/ml
4	0,9	30,2
5	0,6	24,4
10	2,2	36,1
12	86,6	1738,1
13	124,4	2598,0
16	11231,9	6228,4

Table 2.
Quantitative gliotoxin detection of selected fungal culture supernatants in Sabouraud respectively Czapek medium
LOQ (limit of quantification: 10 ng/ml)
4: *Penicillium citrinum*
5: *Penicillium spp.*
10: *Aspergillus ochraceus*
12, 13, 16: *Aspergillus fumigatus*



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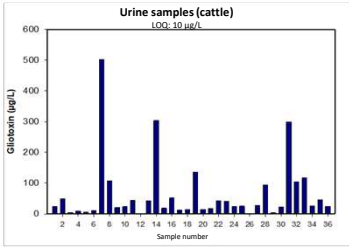

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Figures C, D, E.
Quantitative gliotoxin detection in faecal samples of cattle (Fig. C) and horses (Fig. D) as well as urine samples of cattle (Fig. E).

CONCLUSIONS

- GT detection *in vivo*
  Note: ELISA as detection method in need of confirmation by using a second method (e.g. HPLC-MS) + optimisation of sample preparation
- *Aspergillus fumigatus*: strongest GT producer of the selected fungi
- *Candida sp.*: no GT production detected (consensus with literature)
- Different composition of Czapek and Sabouraud liquid medium (dextrose vs. sucrose + peptons) → stronger stimulation of GT production within Czapek medium

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