

# Time Related Fungal Contamination of Animal Cage Beddings

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## Summary

The purpose of the study was to measure the extent of fungal contamination in laboratory animal cage beddings over time. The material was analysed for the content of fungal enzyme N-acetylhexosaminidase and the fungal cell wall agent 1,3- $\beta$ -glucan at 0-7 days after use. In some cages the values were increased above baseline already at 3 days and at 7 days practically all beddings showed a fungal contamination. It is suggested that the fungal enzyme test can be used for bedding quality control purposes and to monitor fungal contamination in animal cages to prevent pulmonary and other pathologies.

## Introduction

Microbes in the environment may cause effects on the lungs in terms of infection or inflammation due to specific microbial cell wall agents (MCWA) (Fogelmark *et al.*, 1991). Examples of such MCWA are endotoxin in Gram-negative bacteria and 1,3- $\beta$ -glucan, chitin, and sugars such as mannan in fungi. Previous experience has demonstrated that increased levels of MCWA in bedding in animal cages are related to lung pathology among the animals in terms of an abnormal accumulation of cells in the lung tissue (Ewaldsson *et al.*, 2001). The microbial contamination of bedding materials is dependent on source characteristics but environmental conditions in the cage are also important.

Fungal growth will take place in bedding materials due to high material humidity. MCWA from fungi such as 1,3- $\beta$ -glucan induce effects on the immune system (Wan *et al.*, 1999; Rylander and Lin 2000) and act synergistically on the effects of other inflammatory agents such as bacterial endotoxin

(Fogelmark *et al.*, 1994).

Fungal contamination is usually detected by counting the number of spores, by culturing for number or species determination or by determining the specific amount of a fungal cell wall agent such as ergosterol or 1,3- $\beta$ -glucan. An alternative method is to determine specific enzymes present in fungi (Reeslev *et al.*, 2003, Madsen 2003). The enzyme method is less complicated from a technical point of view: it does not require sterility, and the results are obtained within a few hours.

The purpose of the present study was to measure the extent of fungal contamination in bedding in animal cages housing rats at different times of occupation. The content of 1,3- $\beta$ -glucan and fungal enzyme levels were used as markers for the contamination.

## Materials and Methods

Rats were housed 5 per cage (floor area 2065 cm<sup>2</sup>) on woodchip bedding (Lignotel™) with food and water ad lib. Room temperature was maintained in the range 21±2°C, relative humidity 55±10% with 20 air changes/hour, in accordance with UK Home Office requirements under the Animals (Scientific Procedures) Act 1986.

Bedding samples were randomly collected at de-

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livery and at 3, 4 and 7 days after housing rats in the cages. The samples were analysed using a standardized technique involving extraction with 10 ml pyrogene-free water for 10 minutes. One ml of the extract fluid was diluted and analysed.

The amount of the fungal enzyme N-acetylhexosaminidase (NAHA) was determined using a commercially available kit (Mycometer, Copenhagen, Denmark) and according to standard operating procedures (SOP). The extract from the bedding was placed in a tube containing 2 ml of buffer with a NAHA substrate. After 30 minutes of incubation at ambient temperature, a developer was added and the fluorescence was measured in a Picofluor fluorometer (Turner Designs, Sunnyvale, CA, USA). The fluorescence units read were taken as enzyme activity units.

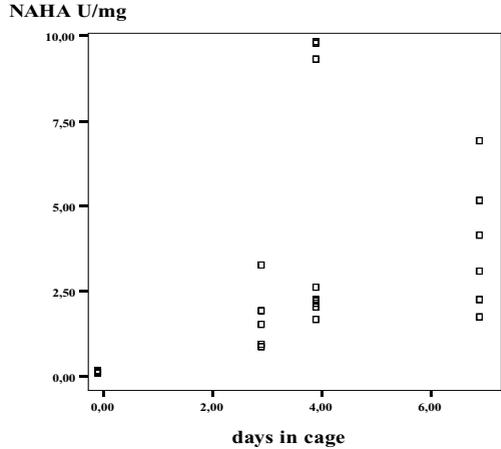
One ml of 3 N NaOH was added to the remainder of the extract and after 10 minutes it was analyzed for 1,3-β-glucan with the Limulus lysate test, using a specific lysate according to the instructions of the manufacturer (Ass Cape Cod, Mass, USA). The endpoint was the kinetics of the colour reaction initiated by 1,3-β-glucan. The results were related to a standard preparation and expressed as amount of 1,3-β-glucan per mg material.

**Results**

There was a significant relationship between the amount of 1,3-β-glucan and NAHA in the different samples (Spearman 0.541, p=0.006).

The amounts of NAHA and 1,3-β-glucan in bed-

ding samples taken at different days are shown in table 1.



**Figure 1.** Fungal enzyme NAHA levels in bedding samples after different days in cage.

The values at day 0 were low. On subsequent days there was a gradual increase in the amount of NAHA. At days 4 and 7 all values were higher than on day 0.

**Discussion and Results**

The main result from the study was the increased amount of fungal enzyme and 1,3-β-glucan on day 7 in all samples of bedding. There was a reasonable

**Table 1.** Amount of NAHA (U/mg) and 1,3-β-glucan (median and range) in bedding samples after different days in cage.

Day	samples n	NAHA		1,3-β-glucan	
		M	r	m	r
0	4	0.02	0-0.1	2.6	1.2-4.3
3	5	1.8	0.8-3.2	18.4	2.6-32
4	9	2.1	1.9-9.7	11.0	4.4-26
7	6	3.0	1.6-6.8	17.3	2.5-48

The results for NAHA are illustrated in Figure 1.

relationship between 1,3- $\beta$ -glucan and fungal enzyme levels. There are, however, some limitations to the study.

The results are based upon a fairly small number of observations but the results at day 7 were very consistent. No detailed control was performed of housing conditions and the differences in the degree of fungal contamination at days 3 and 4 could not be explained. Furthermore the determination of fungi only comprises part of the microbial exposure to laboratory animals. Bacteria and viruses transmitted from animal to animal are important sources for pathological changes in the animals, which will influence the well being of the animals as well as results from experiments. The conditions in the bedding materials with increased levels of humidity during storage favour the growth of fungi. In view of the effects of fungal cell wall agents on the immune system, this contamination is probably an important part of the microbial risk in the cage environment.

The amounts of NAHA and 1,3- $\beta$ -glucan had already increased in some samples already on day 3. Within the limits of the study it was not possible to relate this increase to any specific housing condition. Apart from differences in the quality of the bedding material, the difference is probably due to housing conditions favouring the growth of microbes such as number of animals per cage or wetting of the bedding.

Regarding the analysis used, the Limulus test is very sensitive and specific for 1,3- $\beta$ -glucan and the results indicate the presence of mould cell biomass. On the other hand the test is expensive and quite cumbersome. The determination of NAHA is cheaper and simpler and the results are available within hours. In this study there was a modest relation between these measures but a certain scatter in the relation. This would be expected as the two methods express different constituents in the fungal cell walls. As the results regarding the increase in levels with number of days were similar, both methods are suitable for screening of microbial contamination of bedding materials, both as material quality

control and as follow-up during animal housing.

Overall the results suggest that an increase in the fungal contamination of bedding materials takes place already after a few days in the cage. In a previous study it was shown that agitation of bedding materials contaminated with 1,3- $\beta$ -glucan may generate airborne levels considerably above those known to cause inflammation (Ewaldsson *et al.*, 2002). 1,3- $\beta$ -glucan has a number of pro-inflammatory effects, depending on its structure and solubility in water (Young and Castranova, 2005). It acts synergistically with endotoxin (Fogelmark *et al.*, 1994). Water-soluble and non-soluble forms from *Candida albicans* increase the production of several inflammatory cytokines such as IL-1 and TNF $\alpha$  (Ishibashi *et al.*, 2005). Cell infiltration and a Th2 skewed immunological response pattern are other features of inhalation exposure to 1,3- $\beta$ -glucan (Wan *et al.*, 1999).

In conclusion, the results from this pilot study suggest that measurements of a fungal enzyme could be used as a rapid screening method to survey bedding fungal contamination both at delivery and when used and thus assess the risk of pulmonary pathology in animals. Further studies are required to test this hypothesis.

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