



Fungi in Aerosols of Hay Associated with Respiratory Distress in Dairy Cattle

W. G. Sorenson, Paul D. Siegel, Stephen A. Olenchock

National Institute for Occupational Safety and Health, 944 Chestnut Ridge Road,
Morgantown, West Virginia 26505, USA

&

John J. May, David S. Pratt

New York Center for Agricultural Medicine and Health, Cooperstown, New York,
USA

(Received 4 May 1992; revised version accepted 24 July 1992)

ABSTRACT

*Three samples of hay associated with an outbreak of bovine respiratory disease were examined for possible etiological agents. The total numbers of viable microorganisms in the samples ranged from 10^4 to 10^6 colony-forming units/mg dust. Mycological studies on these samples revealed that the predominant fungi were members of the genus *Aspergillus* with fewer numbers of *Penicillium*, *Phoma*, and *Aureobasidium*. The predominant *Aspergilli* observed were members of the xerophilic *A. glaucus* group. Counterimmunoelectrophoretic analysis demonstrated that one of the hay samples (sample 3) contained material which reacted with all except two of the bovine sera tested and with serum from both exposed and unexposed human subjects.*

INTRODUCTION

Agricultural dust contains a myriad of substances and is known to cause adverse respiratory effects in agricultural workers and other individuals

exposed to high concentrations of dust (Pratt & May, 1984; doPico, 1986; May *et al.*, 1986). The bedding chopper is a mechanical device which cuts hay into short lengths and blows it into cattle stalls to be used as bedding by dairy cows. This operation creates a considerable dust cloud containing high levels of microorganisms and other materials, e.g. endotoxin (Pratt *et al.*, 1990). A recent study of endotoxin air concentrations associated with these choppers demonstrated levels as high as 27096 EU/m³ (Pratt *et al.*, 1990). Endotoxin is also commonly found in high levels in cotton dust associated with adverse respiratory responses (Castellan *et al.*, 1987). The present study reports the results of an examination of three hay samples from a barn in which both the farmer and the dairy cows had symptoms associated with the use of a bedding chopper. The presence of endotoxin and histamine were previously demonstrated in these samples (Siegel *et al.*, 1991). The cows displayed asthma-like symptoms. The farmer who operated the bedding chopper had no respiratory symptoms during the day, but complained of congestion at night. Hay extracts were examined for the presence of bacteria and fungi and respirable dust clouds were also generated from these samples to study the presence of microorganisms introduced into the air.

MATERIALS AND METHODS

Hay samples

Three samples of hay from barns in which 50% of the cows displayed wheezing and coughing spells were obtained from farms located near Cooperstown, NY. Sample 1 consisted of hay and hay chaff which was the accumulation of approximately 5 years between the floor of the mow and the ceiling of the milking floor of the barn. This hay had been exposed to leakage from the barn roof, it showed obvious evidence of decay, and was originally thought to be the cause of the problem in one of the cows because chaff, from this hay, had fallen directly on her head. Sample 2 was newly purchased from a local source. This sample had been refused by a horse farm because it was too dusty for horses but the cows did not refuse to eat it. Sample 3 was estimated to be approximately 1 year old and probably had got wet during storage because the roof above it leaked badly. This was replaced soon after the new owner moved to the farm. All the samples were sent to Morgantown for study.

The hay samples were chopped and sieved through a 20 mesh stainless

steel screen before being introduced into the aerosol generator. The system described by Sorenson *et al.* (1987) was used for aerosol generation and analysis. Respirable-sized dust particles were generated from 10–20 g portions of hay samples in a miniature version of the Pitt-3 acoustical dust generator (Weyel *et al.*, 1984). This minigenerator has a Plexiglass tube (approximately 38 cm high with an inner diameter of approximately 12 cm) with vibration provided by rubber dam material over a 6.3 cm speaker, powered at 60 Hz and 3V by an autotransformer. Filtered air was introduced into the generator at 2 liters/min (1pm) and mixed with filtered room air prior to being introduced into a 20 liter glass carboy used as a settling chamber. Exhaust air flow was maintained at 7.4 lpm by three critical orifices sealed into Nalgene tubing with silicone adhesive and calibrated by the soap bubble meter method (Levy, 1964). Respirable dust was collected at 1.7 lpm through a 10 mm cyclone (Mine Safety Appliances Co., Pittsburgh, PA) onto an in-line, preweighed 37 mm 0.45 μm HA filter (Millipore, Bedford, MA). Total dust concentrations were determined by collecting air at 4.25 lpm through an in-line 37 mm 0.45 μm HA filter. Total and respirable dust concentrations were determined gravimetrically. Samples for microbial analysis were collected on in-line, sterile 37 mm polycarbonate filters (pore size = 0.2 μm) at a flow rate of 1.5 lpm for 20–30 min to minimize possible viability loss due to desiccation. All components of the system were either replaced or disinfected with 70% isopropanol between samples in order to reduce or eliminate the possibility of carry-over of microorganisms from sample to sample.

For estimation of the concentration of bacteria and fungi in aerosolized hay, the respirable-sized dust particles were removed from the polycarbonate filters by the method of Palmgren *et al.* (1986). In brief, 1.5 ml of sterile water containing 0.1% (w/w) of Tween 80 was added through the outlet connection of the cassette to saturate the cellulose back-up pad and then 5 ml of the same solution was added through the inlet connection. The cassette was replugged and shaken for 15 min on a shaking table. After shaking, portions of the fluid on the inlet side of the filter were used for estimation of total microorganisms by scanning electron microscopy and the remainder of the fluid was used for dilution plating to determine the number of viable microorganisms present. Undiluted samples for SEM were collected on a second filter, dehydrated through a graded series of ethanol, and transferred into graded amyl acetate-ethanol solutions to 100% amyl acetate. Samples were then dried using a Denton DCP-1 apparatus. Samples were mounted onto carbon planchets, placed on aluminium SEM stubs, and coated with gold-

paladium (200 Å). Coating of the samples was carried out using a Polaron E5100 Series III sputted coater. SEM examination was performed using an ETEC Autoscan SEM operating at 20 kV. The number of spores or bacterial cells was counted from at least 10 fields of view at a magnification of 3000× (Pasanen *et al.*, 1989). Serial 10-fold dilutions were made, and 0.1-ml portions were plated in triplicate on the following agar media: tryptic soy agar (TSA) for total bacteria, eosin methylene blue agar (EMB) for Gram-negative bacteria, half-strength tryptic soy agar (TSA/2) for thermophilic actinomycetes, and rose bengal-streptomycin (RBS) and dichlor-glycerol agar (DG18) for fungi. DG18 (Hocking & Pitt, 1980) was originally formulated for the enumeration of xerophilic fungi, which are known to be common contaminants of cereals. This medium has been proved to be useful for the enumeration of other fungi as well (Hocking & Pitt, 1980). The agar plates were incubated at 28°C for 5–7 days (RBS and DG18), 35°C for 2 days (TSA and EMB), 45°C for 5–7 days (RBS), and 55°C for 7 days (TSA/2). After incubation, colonies were counted in order to determine the concentration of viable organisms per sample.

Because the aerosol was generated in an artificial system which may or may not simulate actual dust concentrations in the workplace, concentrations of microorganisms in the aerosol are expressed in colony-forming (CFU) units per milligram of dust rather than CFU/m³. In order to estimate the frequency of individual organisms in the aerosols, representative plates of each medium were selected and each colony on these plates was transferred to agar slants for further study. Fungi were identified on the basis of colony and microscopic morphology, and, in the case of *Penicillium* species, on their growth rates on specific media (Pitt, 1979). Raper & Fennell (1965) and Pitt (1979) were used for the identification of *Aspergillus* and *Penicillium* isolates respectively.

Serum samples were collected from 12 exposed and six unexposed cows and from one exposed farmer and from one unexposed human volunteer. Ten of the exposed cows were sick. Each serum was tested for specific precipitating antibody to extracts from the three hay samples by counterimmunoelectrophoresis (CIEP) by the procedure of Gordon *et al.* (1971). A positive control was used with each CIEP plate. Bulk samples were extracted in sterile pyrogen-free water by rocking for 60 min at ambient room temperature and then centrifuging at 1000 g for 10 min. Supernatant fluids were stored at 85°C before use. Positive controls were used with each CIEP plate and consisted of known *Aspergillus fumigatus* antigens from Greer Laboratories Inc. (Lenoir, NC).

RESULTS

The total numbers of viable microorganisms detected in these samples are summarized according to the broad category of organism in Table 1. The numbers of viable microorganisms ranged from *c.* 10^4 to *c.* 10^6 CFU/mg of dust with the highest concentrations found in Sample 3. Thermophilic actinomycetes were not detected in these samples. In general, numbers of viable fungi and bacteria were comparable in Samples 1 and 3, whereas in Sample 2, fungi were found in *c.* 40- to 50-fold greater numbers than bacteria. Of the viable microorganisms observed in these samples, the mesophilic fungi were found in the greatest numbers, especially members of the xerophilic *Aspergillus glaucus* group (anamorphs of the genus *Eurotium* Link ex Fr., *E. rubrum*, *E. chevalieri*, and *E. amstelodami*). Although the numbers of fungi from each sample were nearly equal on the two media (Table 2), the species observed were not. For example, *E. rubrum* and *E. chevalieri* were not isolated from RBS, even though equal portions of the same dilutions were plated on both media at the same time. On the other hand, *E. amstelodami* was isolated from both media in roughly equal numbers. *Aspergillus versicolor*, although not observed in hay 2, was a major component of the mycota of hay 1 and hay 3. *Penicillium aurantiogriseum* was found only in hay 3 and *Penicillium spinulosum* only in hay 1.

Thermotolerant fungi in these hay samples consisted predominantly of *Aspergillus fumigatus* with smaller numbers of *Aspergillus nidulans* and *Absidia corymbifera*. Total numbers of these fungi were not exceptionally high (Table 1). Certain of the fungi observed on RBS, e.g. *Aureobasidium pullulans*, were not observed on DG18. The numbers of organisms observed by SEM on Nucleopore filters were one to three orders of magnitude higher than the numbers of viable organisms (Table 3).

TABLE 1
Viable Microorganisms from Aerosolized Hay (CFU/mg of dust)

	Hay Sample 1	Hay Sample 2	Hay Sample 3
Total bacteria	1.2×10^4	1.7×10^3	2.3×10^5
Gram-negative bacteria	ND	ND	4.5×10^6
Mesophilic fungi	1.2×10^4	8.5×10^4	1.1×10^6
Xerophilic fungi	1.0×10^4	7.2×10^4	1.1×10^6
Thermotolerant fungi	2.9×10^2	9.2×10^1	8.9×10^2

ND: not detected.

TABLE 2
Mesophilic Fungi from Aerosolized Hay (CFU/mg of dust)

	RBS agar			DG18 agar		
	Hay Sample 1	Hay Sample 2	Hay Sample 3	Hay Sample 1	Hay Sample 2	Hay Sample 3
<i>Aspergillus versicolor</i>	8×10^3	ND	8.5×10^5	1.9×10^3	ND	3.1×10^5
<i>Aureobasidium pullulans</i>	ND	ND	5.0×10^5	ND	ND	ND
<i>Eurotium amstelodami</i>	4×10^2	8.5×10^4	ND	3.7×10^2	6.9×10^4	3.9×10^4
<i>Eurotium chevalieri</i>	ND	ND	ND	3.7×10^2	3.3×10^3	ND
<i>Eurotium rubrum</i>	ND	ND	ND	4.1×10^3	ND	1.6×10^5
<i>Penicillium aurantiogriseum</i>	ND	ND	ND	ND	ND	5.9×10^5
<i>Penicillium spinulosum</i>	2.8×10^3	ND	ND	ND	ND	ND
<i>Phoma</i> sp.	ND	ND	ND	3.7×10^2	ND	ND
Undetermined	8.0×10^2	ND	2.0×10^5	3.0×10^3	ND	4.0×10^4
Total	1.2×10^4	8.5×10^4	1.1×10^6	1.0×10^4	7.2×10^4	1.1×10^6

ND: not detected.

TABLE 3
Total Microorganisms Observed by SEM on Nucleopore Filters (spores and/or cells per mg of dust)

	Hay Sample 1	Hay Sample 2	Hay Sample 3
Bacteria	2.7×10^5 (4.4) ^a	1.1×10^6 (0.2)	9.8×10^6 (2.4)
Fungi	1.6×10^6 (0.8)	5.2×10^6 (1.5)	6.9×10^6 (15.9)

^aNumbers in parentheses represent the viable counts expressed as a percentage of the counts observed by SEM.

Counterimmunoelectrophoretic analysis revealed that sera obtained from 8 of 10 sick cows, 2 of 2 healthy cows from the same barn, and from 6 of 6 cows from other farms produced precipitin bands with Sample 3. This same sample reacted with serum from both exposed and unexposed human subjects. No precipitin bands were observed with Samples 1 or 2 (data not shown).

DISCUSSION

Total viable microorganisms in these samples were comparable to the levels reported in other studies (Pratt & May, 1984; May *et al.*, 1986; Dutkiewicz *et al.*, 1989) except that Gram-negative bacteria were not

detected in hay 1 or hay 2 and represented less than 2% of the total viable bacteria in hay 3. In spite of the respiratory disease symptoms observed in the dairy cattle, the numbers of thermophilic actinomycetes commonly associated with farmer's lung disease (FLD) were low. This suggests (1) that the etiologic agents were present in sufficient concentration to elaborate symptoms in the absence of large numbers of irrelevant microorganisms, or (2) that other, as yet undefined, substances were present in the hay which are the etiologic agents.

The most striking group of fungi in these samples were members of the *A. glaucus* group. All are xerophilic fungi common on cereal grains (Raper & Fennel, 1965; Ito *et al.*, 1973; Le Bars & Escoula, 1973; El-Sharouny *et al.*, 1988). According to the International Code of Botanical Nomenclature, the correct generic name for these fungi is *Eurotium* because of the presence of the asci and ascospores. *Eurotium umbrosum*, a member of this group, has been reported to be particularly abundant in hays associated with FLD in Finland and it reacted with most sera from farmer's lung patients in precipitin tests there (Terho & Lacey, 1979). Members of this group are remarkably similar, both morphologically and biochemically. *Eurotium umbrosum* is regarded by some authors (Pitt, 1985; Pitt & Hocking, 1985) as a synonym of *Eurotium herbariorum*, which is closely related to *Eurotium rubrum*. In addition, Blaser (1976) has suggested that both *E. rubrum* and *E. umbrosum* be reduced to synonymy with *E. herbariorum* on the basis of ascospore structure. Although this view is not universal (Kozakiewicz, 1985), it has been suggested that *E. rubrum* and other members of this group be investigated as probable candidate agents of FLD and ODS (Warren, 1981). *Eurotium amstelodami* and *E. rubrum* were reported to be both toxic and common in industrial feed mixtures (Borkowska-Opacka & Truszczynski, 1979). The relative frequency of occurrence of these fungi on RBS and DG18 underscore the value of using a variety of media, each designed to enhance the growth of different groups of fungi when attempting to characterize and enumerate fungi in food stuffs and/or other organic substrates (Samson, 1985).

Although FLD is a hypersensitivity disease in which a high proportion of patients show precipitating antibodies to known agents, positive serological response is not necessarily a feature of ODS (doPico, 1986). The positive CIEP reactions observed in sera of both cows and humans to extracts of hay Sample 3 is probably due to the presence of common antigens to which virtually everyone has been exposed. Nicolet *et al.* (1972) and Pirie *et al.* (1972) reported that c. 80% of exposed cattle had precipitins to moldy hay antigens and/or *Microspora faeni* antigens.

Siegel *et al.* (1991), examined these hay samples for the presence of

endotoxin and histamine. Endotoxin was found in the bulk hay as well as the aerosol samples produced in the Pitt 3 generator. Endotoxin in the bulk hay ranged from 93 to 6138.3 EU/mg hay and the endotoxin concentrations in the aerosol ranged from 474.6 to 1473.6 EU/mg dust. There was little difference between the respirable and total dust concentrations from the aerosol, suggesting that almost the entire dust cloud was of respirable size ($<10\ \mu\text{m}$). Histamine was demonstrated in the bulk hay and Samples 2 and 3 (0.5 and 0.175 ng/mg, respectively) but not in Sample 1. The presence of histamine in the absence of creatinine suggests that histamine was not of animal origin and raises the possibility that it was produced by microorganisms (Siegel *et al.*, 1991). Although the Pitt 3 generator does not necessarily simulate dust generation during the bedding chopper operation, it generates samples containing respirable size particles, it provides a laboratory model of what could be aerosolized from these materials, and it demonstrates that the microbial contaminants of hay can be easily aerosolized.

These data demonstrate that dusts generated from hay samples associated with an outbreak of respiratory illness in cows contained high levels of fungi and bacteria. The fungi were predominantly of the *A. glaucus* group, previously reported to be associated with FLD in humans.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the help of Mrs Diane Schwegler-Berry with the scanning electron microscopy.

REFERENCES

- Borkowska-Opacka, B. & Truszczynski, M. (1979). The occurrence of toxigenic fungi in industrial feed mixtures. *Pol. Arch. Weter.* **21**, 51-64.
- Blaser, P. (1976). Taxonomische und physiologische Untersuchungen über die Gattung *Eurotium* Link ex Fries. *Sydowia*, **28**, 1-49.
- Castellan, R. M., Olenchock, S. A., Kinsley, K. B. & Hankinson, J. L. (1987). Inhaled endotoxin and decreased spirometric values: An exposure-response relation for cotton dust. *New Engl. J. Med.*, **317**, 605-10.
- doPico, G. A. (1986). Report on diseases. *Am. J. Ind. Med.*, **10**, 261-5.
- Dutkiewicz, J., Olenchock, S. A., Sorenson, W. G., Gerencser, V. F., May, J. J., Pratt, D. S. & Robinson, V. A. (1989). Levels of bacteria, fungi, and endotoxin in bulk and aerosolized corn silage. *Appl. Environ. Microbiol.*, **55**, 1093-9.
- El-Sharouny, H. M. M., Moubasher, A. H. & Nassar, M. S. (1988). Mycoflora

- associated with dry dates in Upper Egypt. II. Osmophilic fungi and test of osmophilic ability. *Qatar Univ. Sci. Bull.*, **8**, 59–68.
- Gordon, M. A., Almy, R. E., Greene, C. H. & Fenton, J. W. (1971). Diagnostic mycoserology by immunoelectrophoresis: A general, rapid, and sensitive microtechnic. *Am. J. Clin. Pathol.*, **56**, 471–4.
- Hocking, A. D. & Pitt, J. I. (1980). Dichloran-glycerol medium for enumeration of xerophilic fungi from low-moisture foods. *Appl. Environ. Microbiol.*, **39**, 488–92.
- Ito, H., Iizuka, H. & Sato, T. (1973). Identification of osmophilic *Aspergillus* isolated from rice and their radiosensitivity. *Agric. Biol. Chem.*, **37**, 789–98.
- Kozakiewicz, Z. (1985). Solutions to some problems in *Aspergillus* taxonomy using the scanning electron microscope. In *Advances in Penicillium and Aspergillus Systematics*, ed. R. A. Samson & J. I. Pitt. NATO Advanced Science Institute Series, Plenum Press, London.
- Le Bars, J. & Escoula, L. (1973). The fungi of dry fodder. I. Inventory and frequency of species. *Ann. Rech. Vet.*, **4**, 273–82.
- Levy, A. (1964). The accuracy of the bubble meter method for gas flow measurements. *J. Sci. Instr.*, **41**, 449.
- May, J. J., Pratt, D. S., Stallones, L., Morey, P. R., Olenchock, S. A., Deep, I. W. & Bennet, G. A. (1986). A study of silo unloading: the work environment and its physiologic effects. *Am. J. Ind. Med.*, **10**, 318.
- Nicolet, J., Haller, R. de & Herzog, J. (1972). Serological investigations of a bovine respiratory disease ("Urner pneumonie") resembling farmer's lung. *Infect. Immunity*, **6**, 38–42.
- Palmgren, U., Strom, G., Malmberg, P. & Blomquist, G. (1986). The nucleopore filter method: a technique for enumeration of viable and nonviable airborne microorganisms. *Am. J. Ind. Med.*, **10**, 325–7.
- Pasanen, A. L., Kalliokoski, P., Pasanen, P., Salmi, T. & Tossavainen, A. (1989). Fungi carried from farmers' work into farm homes. *Am. Ind. Hyg. Assoc. J.*, **50**, 631–3.
- Pirie, H. M., Dawson, C. O., Breeze, R. G., Selman, I. E. & Wiseman, A. (1972). Precipitins to *Micropolyspora faeni* in the adult cattle of selected herds in Scotland and north-west England. *Clinical Allergy*, **2**, 181–7.
- Pitt, J. I. (1979). *The Genus Penicillium and its Teliomorph States Eupenicillium and Talaromyces*. Academic Press, New York.
- Pitt, J. I. (1985). Nomenclatorial and taxonomic problems in the genus *Eurotium*. In *Advances in Penicillium and Aspergillus Systematics*, ed. R. A. Samson & J. I. Pitt. NATO Advanced Science Institute Series, Plenum Press, London.
- Pitt, J. I. & Hocking, A. D. (1985). *Fungi and Food Spoilage*. Academic Press, Sydney, Australia.
- Pratt, D. S. & May, J. J. (1984). Feed-associated respiratory illness of farmers. *Arch. Environ. Health*, **39**, 43–8.
- Pratt, D. S., May, J. J., Reed, C. E., Swanson, M. C., Campbell, A. R., Piacitelli, L., Olenchock, S. & Sorenson, W. (1990). Massive exposure to aeroallergens in dairy farming: radioimmunoassay results of dust collection during bedding chopping with culture confirmation. *Am. J. Ind. Med.*, **17**, 103–4.
- Raper, K. B. & Fennell, D. I. (1965). *The Genus Aspergillus*. The Williams & Wilkins Co., Baltimore.

- Samson, R. A. (1985). Occurrence of molds in modern living and working environments. *Eur. J. Epidemiol.*, **1**, 54-61.
- Siegel, P. D., Olenchock, S. A., Sorenson, W. G., Lewis, D. M., Bledsoe, T. A., May, J. J. & Pratt, D. S. (1991). Histamine and endotoxin contamination of hay and respirable hay dust. *Scand. J. Work Environ. Health*, **17**, 276-80.
- Sorenson, W. G., Frazer, D. G., Jarvis, B. B., Simpson, J. & Robinson, V. A. (1987). Trichothecene mycotoxins in aerosolized conidia of *Stachybotrys atra*. *Appl. Environ. Microbiol.*, **53**, 1370-5.
- Terho, E. O. & Lacey, J. (1979). Microbiological and serological studies of farmer's lung in Finland. *Clin. Allergy*, **9**, 43-52.
- Warren, C. P. (1981). Respiratory disorders in Manitoba cattle farmers. *Can. Med. Assoc. J.*, **125**, 41-6.
- Weyel, D. A., Ellakkani, M., Alarie, Y. & Karol, M. (1984). An aerosol generator for the resuspension of cotton dust. *Toxicol. Appl. Pharmacol.*, **76**, 544-7.