ELECTRONIC PAPER

Microbiological evolution of hay and relapse in patients with farmer's lung

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Background: Recent studies in France have shown that *Absidia corymbifera* and, to a lesser degree *Eurotium amstelodami* and *Wallemia sebi*, play a role in farmer's lung disease (FLD), but that *Saccharopolyspora rectivirgula*, classically incriminated, does not. Little is known about farmers' reactions to these fungi or the circumstances which lead to exposure.
Aims: To investigate the conditions which favour the development of these microorganisms in hay and to analyse the relation between their concentration and the risk of occurrence of FLD.
Methods: Sequential microbiological analyses of each batch of hay stored in 10 farms at risk for FLD and a serological survey of 10 farmers (five with a past history of FLD).
Results: Exposure to microorganisms varied widely according to farms and periods. These microorganisms usually reached a peak in January and proliferated when harvesting conditions favoured excessive humidity in hay (rain during harvest, soil in the hay). Three of the five FLD patients presented with FLD respiratory recurrence and positive serology for *A corymbifera* during the winter (2000−01), after exposure to a significantly higher amount of *A corymbifera* than other farmers. Similar, but less significant, results were found for *E amstelodami* exposure, but not with *W sebi*.
Conclusions: Results contribute to confirming *A corymbifera* as a major aetiological agent of FLD in Doubs, and encourage further studies with a view to implementing preventive measures.

Farmer's lung disease (FLD) is a immunological reaction to bacterial and/or fungal products found in hay handled during the indoor feeding season,[1] especially in cold and rainy areas.[2] The microbial agent classically said to induce FLD is a thermophilic actinomycete: *Saccharopolyspora rectivirgula* (*Micropolyspora faeni*).[3] In the Doubs, a region in the east of France, *S rectivirgula* has very rarely been isolated[4] despite a high prevalence of FLD, ranging from 2% to 4% of farmers.[5] Moreover, a previous serological study suggested that most farmers in the Doubs had never been exposed to this microorganism.[6] A prospective microbiological and immunological study recently conducted to identify the putative cause of FLD in eastern France showed that sera collected in FLD patients specifically reacted with some moulds, including *Absidia corymbifera* and, to a lesser degree, *Eurotium amstelodami* and *Wallemia sebi,* whereas *S rectivirgula* gave negative results.[7] These results concur with those of a study performed in Finland in which the authors found a level of IgG against *A corymbifera* three times higher in farmers with FLD than in exposed control farmers.[8] Comparable results have also been found with *W sebi* and *Aspergillus umbrosus*, the latter of which, like *E amstelodami*, is a species of the *Aspergillus glaucus* group.[9 10] Although these studies contended that *A corymbifera*, and possibly *E amstelodami* and *W sebi*, were involved in the occurrence of FLD in two distinct areas in Europe, very little is known about the conditions which lead to exposure to these fungi. In order to know if these moulds were present during the entire winter fodder season or only at a specific stage of hay maturation, we designed a longitudinal prospective study of 15 farms.

Five of the farms were owned by patients with a history of FLD; the other five were neighbouring farms with very similar farming methods. All were situated in a mountainous area and used traditional farming methods. The last five were modern farms situated in a plains area where meteorological conditions are dryer.

The main objectives of this study were to follow the evolution of *A corymbifera*, *E amstelodami*, and *W sebi* during the storage period and to determine which conditions led to proliferation of these microorganisms. At the same time, we followed the clinical and serological evolution of exposed farmers. Three of the five working farmers with a history of FLD suffered a relapse.

METHODS

Farms

Fifteen farms were selected for this study. Ten of them were considered as farms at risk: five were owned by subjects with a previous history of FLD and the other five, located in the same area, were owned by asymptomatic farmers with similar agricultural practices. These farms were located at an altitude of 830–1100 metres, in a rainy area where the prevalence of FLD is maximal.[5] Their cowsheds and main barns were made of stone and poorly ventilated. Half of these barns were attached to the house. The last five farms, selected as controls, were modern and located at an altitude of 360–580 metres, in a better weather area. Their cowsheds and barns were made of metal, were well ventilated, and were not attached to the house.

Hay sampling

Data related to farming methods was collected during interviews using a standardised questionnaire which included mode of hay packing (bulk or cylindrical bales), storage location (beside or above the cowshed), hay type (first or second crop of hay), and harvesting conditions which could prevent drying. These conditions, defined a priori, were: terrain liable to flood, rain during harvest, and soil in the hay due to numerous tumuli caused by the proliferation of voles. If any of these conditions was indicated for a batch of hay, it was categorised under ''bad harvesting conditions''. A batch was defined as a set of bales or bulk collected from

Main messages

- Microbiological composition and concentrations of hay varied largely within the same farm.
- The main factor of microbiological proliferation in hay was bad harvest conditions (rain during harvest, soil in the hay).
- Microorganisms involved in farmer's lung disease reached a peak in January and February, which lead to a high exposure period for farmers. This peak corresponded to the period when the number of FLD cases in the region was highest.
- Exposure indexes were established to monitor the microbiological environment of farmers.
- For patients with a history of farmer's lung disease, high exposure to *Absidia corymbifera* and/or *Eurotium amstelodami* lead to relapses. New arguments are provided for considering *A corymbifera* and possibly *E amstelodami* as causative agents of farmer's lung disease and to exempt *Wallemia sebi* in the region.

Policy implications

- An early microbiological analysis of hay should be performed (in November) to anticipate batches of hay which will contaminate with higher concentrations of pathogen microorganisms. Hay storage and distribution management can then be improved, reducing the risk of exposition.
- Other techniques (for example, using artificial drying) can be selected and further studies aimed at preventing humidity in hay at harvest period carried out.
- Farmers should be sensitised to risks associated with a high exposure to pathogen microorganisms, even with respect to manipulating low quantities of highly contaminated hay.

the same meadow at the same period and harvested and stored using the same procedure.

Samples were collected three times during the indoor feeding season (November 2000, January and March 2001) from farms at risk and once (in January) from modern farms. For each batch, two samples were collected in sterile bags, using a standardised method, and the mean value of the number of colonies obtained from cultures was taken into account for analysis in colony forming units per gram of hay (cfu/g). Each sampling was associated with a measure of relative humidity using a mobile humidometer (Wile 25, Tripette & Renaud Agro, Villeneuve-la-Garenne, France).

Microbiological analysis

Each sample was frozen at −18°C overnight to kill the mites. Samples were weighed, rinsed with 20 ml of sterile distilled water, shaken vigorously for one minute, and cultured on Petri dishes. Samples were cultured on five culture media as follows: dichloran-glycerol (Oxoid, Unipath, Basingstoke, UK) with 0.5% chloramphenicol (Merck, Darmstat, Germany) at 30°C for mesophilic mould isolation; Actinomycete isolation agar Bacto medium (Difco, Detroit, MI) at 30°C for mesophilic actinomycetes and at 52°C for thermophilic actinomycetes; 3% malt agar (Oxoid, Unipath, Basingstoke, UK) with 10% salt and 0.5% chloramphenicol, at 20°C for osmophilic fungal species; and R8 medium according to Amner and colleagues at 52°C to detect *Sacharomonospora viridis*.[11] The number of cfu per plate was counted after three and seven days of incubation.

Exposure indexes, representing a theoretical amount of inhalable spores, were calculated every two months for *A corymbifera*, *E amstelodami*, and *W sebi* with the following formula: $I = (H*\Sigma(Xi*Ti))/V$, with Xi = the concentration of each microorganism in every batch of hay handled during the two month period (in cfu/g), Ti = the weight of each batch handled during this period (in grams), H = the number of hours of exposure during this period, and V = the cowshed volume (in cubic metres).

Serological analysis

Serological tests were performed only for farmers working on farms at risk and already treated at the time of a previous study:[7] one at the beginning of the indoor feeding season (October) and one at the end (April). Serum precipitins were measured by electosyneresis on cellulose acetate (Sartorius, Goettingen, Germany) according to a previously described procedure using antigens obtained from species isolated from fodder.[7] Informed written consent was obtained from each subject. The protocol was approved by the local review board for research involving human subjects.

Statistical analysis

The batches which were sampled three times were used to measure the evolution of microorganism concentration and relative humidity during storage (n = 48). The batches which were sampled twice were used to measure harvesting and hay packing conditions leading to the proliferation of microorganisms (n = 71).

Environmental data are known not to show normal distribution. In this case, classic linear models are usually more likely to generate both type I and II errors. Moreover, logarithmic transformation often fails to normalise these right skewed distributions. Consequently, a non-parametric Mann and Withney test (test U) was used to correlate continuous variables (microorganism concentrations and hygrometric measures) and qualitative variables (farm type, harvest, and hay packing conditions). We used Statview 5.0 software (SAS, North Carolina, USA). Probability values less than 0.05 were considered significant.

RESULTS

The number of sampled batches per farm varied from 5 to 15. One hundred and ninety five batches were collected from farms at risk: of the 76 collected in November, 71 were still available for sampling in January and 48 in March. Thirty six samples were collected from modern farms in January and were used to compare the proliferation of microorganisms between modern farms and those at risk.

Microflora of hay and its evolution during feeding season

More than 24 microorganisms were isolated on the 15 farms. Six of them accounted for more than 75% of the total microorganism count: *E amstelodami*, *W sebi*, *A corymbifera*, mesophilic and thermophilic *Streptomyces* spp, and *S viridis*. Others species, listed by percentage of positive samples, were: thermophilic actinomycetes (61%), *Aspergillus umbrosus* (43%), *Cladosporium* spp (30%), *Aspergillus nidulans* (26%), *Penicillium* spp (22%), *Aspergillus fumigatus* (20%), *Aspergillus ochraceus* (18%), *Alternaria* spp (17%), *Aspergillus niger* (11%), *Aspergillus flavus* (8%), *S rectivirgula* (7%), *Rhizopus* spp (7%), *Aspergillus versicolor* (6%), *Mucor* spp.(4%), *Scopulariopsis*

Table 1 Mean concentration* for the main microorganisms isolated in hay samples taken three times from farms at risk (n = 48)

	First series				Second series				Third series			
Microorganism	**Mean (SD)**	**Range**	**%†**	**PS‡**	**Mean (SD)**	**Range**	**%†**	**PS‡**	**Mean (SD)**	**Range**	**%†**	**PS‡**
Fungi												
Absidia corymbifera	4.23 (16.31)	0–110	5.7	44	6.97 (11.91)	0–56	2	39	2.55 (7.51)	0–50	1.1	38
Eurotium amstelodami	17.08 (29.63)	0–140	22.9	16	56.09 (91.53)	0.5–540	15.7	48	20.37 (32.39)	0–151	8.6	46
Wallemia sebi	21.85 (52.63)	0–243	29.1	33	208.24 (660.64)	0–3400	58.4	42	123.20 (311.72)	0–1500	52.1	33
Other fungi	9.23 (23.85)	0–133	12.3		17.86 (41.81)	0–255	5.5		49.14 (132.99)	0–650	20.8	
Actinomycetes												
Mesophilic *Streptomyces*	9.99 (181.92)	0–90	13.4	47	51.32 (131.63)	0–650	14.4	47	22.68 (64.61)	0–400	9.6	42
Thermophilic *Streptomyces*	4.90 (11.49)	0–60	6.6	41	11.53 (25.04)	0–120	3.2	46	4.92 (14.90)	0–81	2.1	37
Saccharomonospora viridis	6.38 (40.41)	0–280	8.5	21	2.41 (61.07)	0–40	0.7	42	4.53 (23.22)	0–160	1.9	34
Saccharopolyspora rectivirgula	0.01 (0.046)	0–0.28	<0.1	3	0.006 (0.047)	0–0.33	<0.1	1	0.063 (0.149)	0–0.6	<0.1	10
Other actinomycetes	0.8 (2.96)	0–17	1.1		2.16 (12.25)	0–85	0.6		0.28 (0.79)	0–5	0.1	

*10^3 cfu/g.
†Percentage of the total microorganism count.
‡Positive sample; number of samples where the microorganism was present for 48 samples.

brevicaulis (2%), *Epicoccum purpuracens* (0.7%), *Blastobotrys nivea* (0.4%), and *Aspergillus terreus* (0.4%).

The concentration of microorganisms in hay was not identical during the indoor feeding season (table 1). In most farms, *A corymbifera*, *E amstelodami*, and mesophilic and thermophilic *Streptomyces* spp reached a peak in January, whereas *W sebi* reached a high concentration in January and stayed at same level. *S viridis* showed two peaks, one in November and the second in March. *S rectivirgula* was rarely isolated in these farms; it was also rarely found in modern farms. In farms at risk, most positive samples for *S rectivirugula* were collected at the end of the indoor feeding season (10 positive samples in March versus only 4 between November and February).

Harvesting and hay packing conditions leading to the proliferation of microorganisms

On modern farms, where samples were collected in January only, the amount of microorganisms was significantly lower than on farms at risk for the same period, particularly for *A corymbifera*, *E. amstelodami*, and *W. sebi* (table 2). On farms at risk, the amount of these microorganisms was related mainly to harvesting conditions. Samples of hay harvested under poor conditions were more contaminated than the others by *A corymbifera* and *E amstelodami* concentrations (table 3).

The impact of hay packing technique seems to be lower in terms of mould proliferation. However, during the storage period, mean relative humidity increased in cylindrical bales from 18.2 to 19.6 degrees and decreased in bulk from 18.9 to 18.5 degrees. Consequently, the difference in relative degrees of humidity between hay packing techniques, which was not significant for the first sampling set ($p = 0.99$), did become significant for the second ($p = 0.02$) and the third ($p<0.01$) sampling sets. This evolution could have consequences on mould proliferation. Indeed, *A corymbifera* concentration was three times higher when hay was stored in high density cylindrical bales rather than in bulk, but the difference was not significant ($p = 0.06$) (table 3).

Observation of three cases of FLD relapse and relation with exposure

Among the five subjects with a previous diagnosis of FLD, three complained of clinical symptoms suggestive of a relapse.

Patient 1, who had been suffering from FLD for eight years, presented with chronic cough and dyspnoea from November to April with an improvement in the spring. He had been exposed to the highest amounts of both *A corymbifera* and *E amstelodami* antigens during the entire indoor feeding season (table 4). He did not notice which batches provoked respiratory symptoms because he usually handled hay from several batches on the same day.

Patient 2 was also exposed to a very high amount of *A corymbifera*, but only in November and December, when he distributed a mouldy batch which he wanted to dispose of (table 4). This batch was highly contaminated with *A corymbifera* (110 000 cfu/g, the highest amount of *A corymbifera* we have ever counted in hay) and *E amstelodami* (80 000 cfu/g). At the same time, he presented with episodes of coughing and fever a few hours after exposure.

Table 2 Mean concentration* of *Absidia corymbifera*, *Eurotium amstelodami*, and *Wallemia sebi* in batches of hay for farms at risk and modern farms

	A corymbifera		*E amstelodami*		*W sebi*	
	Mean	**p value**	**Mean**	**p value**	**Mean**	**p value**
Farms at risk	7.0	0.004	56.0	<0.001	208.0	<0.001
Modern farms	3.6		11.8		49.0	

*10^3 cfu/g.

Table 3 Mean and median* concentrations of *Absidia corymbifera*, *Eurotium amstelodami*, and *Wallemia sebi* in batches of hay in farms at risk according to harvesting and storage criteria (n = 71)

	A corymbifera			*E amstelodami*			*W sebi*		
Criteria	**Mean (SD)**	**Median (range)**	**p value**	**Mean (SD)**	**Median (range)**	**p value**	**Mean (SD)**	**Median (range)**	**p value**
Harvesting conditions									
Good	1.04 (8.9)	1.20 (0–56.5)	<0.001	10.79 (4.5)	8.70 (0.7–100.5)	0.02	9.06 (19.5)	13.33 (0–1706)	0.38 NS
Bad†	5.01 (3.3)	5.75 (0.2–43)		31.19 (4.7)	35.00 (1.6–556)		7.09 (26.3)	5.55 (0–1574)	
Hay packing mode									
Cylindrical bales	2.38 (5.8)	2.48 (0–43)	0.06 NS	18.28 (4.9)	24.55 (0.7–556)	0.14 NS	7.19 (18.6)	8.77 (0–1706)	0.28 NS
Bulk	0.76 (13.2)	1.20 (0–56.5)		9.55 (4.5)	6.84 (0.8–72.5)		12.76 (31.8)	26.91 (0–429.5)	
Storage location									
Beside cowshed	1.87 (8.5)	2.20 (0–56.5)	0.33 NS	16.10 (5.1)	24.38 (0.7–556)	0.60 NS	5.80 (19.8)	10.26 (0–456)	0.03
Above cowshed	1.30 (3.9)	1.90 (0.2–10.2)		12.16 (3.8)	10.89 (2.7–142.2)		70.14 (14.4)	146.56 (0–1706)	
Hay type									
First crop	2.10 (6.3)	2.35 (0–43)	0.34 NS	21.18 (4.6)	30.76 (0.9–556)	0.03	6.90 (30)	12.00 (0–1574)	0.82 NS
Second crop	1.28 (11.1)	1.35 (0–56.5)		8.37 (4.8)	10.09 (0.7–74.6)		12.10 (9.5)	13.30 (250–1706)	

*10^3 cfu/g.
†Conditions which could prevent drying were rain during harvest and soil in the hay due to numerous tumuli caused by the proliferation of voles.
NS, not significant.

Patient 3 was moderately exposed to moulds, except in January when he showed new signs of FLD (cough, dyspnoea, fatigue, and a weight loss of 15 kg). At this time, he was handling 1.5 tons of batches, samples of which he gave to the laboratory investigators. This hay was strongly contaminated by *A corymbifera* and *E amstelodami* (45 000 cfu/g and 168 000 cfu/g, respectively).

In the spring, all three patients presented with positive serology for *A corymbifera*. Two other subjects (subjects 4 and 5 with no history of FLD) were exposed to fairly high amounts of *A corymbifera*, though less than that of subjects 1 and 2, but experienced no symptoms. One of them, however, showed a positive serology in the spring (three arcs with *A corymbifera* antigen) and underwent further medical examination, including spirometry, which revealed no respiratory symptoms or anomaly in respiratory function.

On the whole, exposure indexes to *A corymbifera* measured in the farms and periods when patients presented with symptoms compatible with FLD (periods 1, 2, and 3 for patient 1; period 1 for patient 2; and period 2 for patient 3) were significantly higher than exposure indexes measured in other farms and periods (median 239, range 52–565 versus median 11, range 2–190; p = 0.001). The same tendencies were noted for *E amstelodami*, albeit to a lesser degree (median 777, range 462–2709 versus median 140, range 5–1749; p = 0.01).

Exposure to *W sebi* was also very high on some farms. However, no relation could be established with the occurrence of respiratory symptoms or the appearance of precipitin arcs using specific antigens (data not shown).

DISCUSSION

The amount of microorganisms in hay was significantly lower on modern farms than on farms at risk. On the latter, the main microorganisms reached a peak of proliferation in January. The most contaminated hay had been harvested wet. Hay packing techniques for cylindrical bales may have contributed to the proliferation of microorganisms by maintaining a high humidity level in hay. Three working farmers with a history of FLD suffered a relapse and presented a positive serology for *A corymbifera* during winter after having been exposed to a significantly higher amount of

Table 4 Serologic evolution and clinical features related to intensity of exposure to *Absidia corymbifera* and *Eurotium amstelodami*

		A corymbifera						*E amstelodami*						
		Serology (no. of arcs)		**Exposure indexes†**				**Serology (no. of arcs)**		**Exposure indexes†**				**Clinical features during indoor feeding season**
Farmer no.	**Antecedents of farmer's lung disease**	**Beginning of winter**	**End of winter**	**Period 1**	**Period 2**	**Period 3**	**Total**	**Beginning of winter**	**End of winter**	**Period 1**	**Period 2**	**Period 3**	**Total**	
1	Yes	3*	2*	67	239	565	871	1	3*	690	2709	922	4321	Several attacks from November to April
2	Yes	1	2*	490	38	25	553	3*	0	462	133	140	735	Acute attack in November
3	Yes	3*	4*	8	52	15	75	2	3*	208	777	91	1076	Acute attack in January
4	No	1	1	5	190	32	227	3*	0	21	663	47	731	Asymptomatic
5	No	3*	3*	8	92	14	114	1	1	74	1163	344	1581	Asymptomatic
6	Yes	2*	1	45	20	7	72	1	1	256	509	86	851	Asymptomatic
7	No	1	0	4	44	2	50	1	1	100	1749	5	1854	Asymptomatic
8	No	2*	2*	18	4	10	32	2	2	102	653	279	1034	Asymptomatic
9	Yes	0	0	2	5	12	19	3*	2	20	41	411	472	Asymptomatic
10	No	1	1	4	7	<1	12	0	1	62	1094	556	1712	Asymptomatic

*These results are considered as positive according to thresholds determined in a previous study:[7] two arc theshold with the *A corymbifera* antigen and three arc theshold with the *E amstelodami* antigen.
†Exposure indexes in 10^6 cfu/h/m^3 to each microorganism were calculated for three periods: period 1: November, December; period 2: January, February; period 3: March, April.

this microorganism. Similar, but less significant, results were found for *E amstelodami* but not for *W sebi*.

To perform this study, we decided to monitor the evolution of hay prospectively during the distribution period. This allowed us to quantify exposure by exposure indexes, which include the level of contamination of all batches handled by farmers during the three periods as well as other specific variables, such as work time. This way, it was possible to take into account the putative variability in microorganism populations within batches of hay and working conditions, both principal factors of exposure. We chose not to perform a sequential analysis of the air in the cowsheds because the amount of microorganisms in the air varies considerably in a given cowshed from one day to another, partially as a result of handling different batches of hay. Indeed, a single measure of air contamination at a given time is not representative of exposure for the entire period studied.

In order to heighten the probability of recording the proliferation of moulds in hay, we selected farms either in which FLD episodes had previously been recorded or which were very similar with respect to location, farming methods, and construction. Because these farms were selected, they were not representative of all farms in our region, either for agricultural practice or for microbiological findings. To widen the scope of the investigation, we included five modern farms located in an adjacent area.

The first outcome of this study is the identification of some of the harvesting and storage conditions which lead to the concomitant proliferation of *A corymbifera*, *E amstelodami*, and *W sebi*. Since the 1960s, fewer than 20 microbiological studies have been performed in dairy farms. They measured all airbound microorganisms globally, sometimes in large categories (fungi, actinomycetes, other bacteria, etc). These one-time measures were taken over a short period of time and linked to a specific task (hay and straw handling, drying, unbaling, etc). Hence, they do not reflect the long term evolution of hay.[12–15] In this study, we show that the concentration of the majority of microorganisms in hay increases between October and January, then slowly decreases between January and March. The peak in the middle of winter corresponds to the period when the number of FLD cases was highest in our region. We were able to ascertain the onset of the disease for 24 of the 29 cases of FLD diagnosed and registered in our hospital between 1997 and 2002. For 16 of them, the first symptoms were felt between December and February (data not published). The microbiological evolution of hay was not limited to two periods, as is commonly supposed (harvest flora followed by storage flora after fermentation).[16 17] Regarding relative humidity, hay packed in cylindrical bales became more humid at the end of the winter fodder season, whereas hay packed in bulk became drier. The reasons for this increase in humidity were not clear, but we presume that it was linked to the metabolism of the microorganisms. Gregory and colleagues, reporting in-depth studies of the evolution of hay, attributed the role of *A corymbifera* to furthering the increase of *S rectivirgula*.[18 19] It is worth noting that these studies focused essentially on the two months following harvest. These authors suggested that excess humidity—more than 30% water content in the field—leads to increased heating of the hay as well as a selection of microorganisms. First, thermotolerant fungi (that is, *A corymbifera*) cause biochemical changes and an increase in temperature, which provide the optimum conditions for *S rectivirgula* and other thermophilic actinomycetes to grow. In our area, humidity levels are lower in hay at harvest time (mean 23%, range 18–35%),[20] so it would seem that this process is interrupted or slowed down. This would partly explain why the isolation of *S rectivirgula* occurs later, during the indoor feeding season.

The influence of hay packing techniques or storage building type was not clearly shown in our study, even if cylindrical bale storage was associated with a higher, albeit not significantly higher, level of *A corymbifera* proliferation. The influence of cylindrical bale hay packing techniques has been observed on the proliferation of *S rectivirgula*, also a thermotolerant species.[21] In another study performed in our region, limitation of the proliferation of microorganisms was associated with ventilated bulk.[4]

Secondly, despite the low number of subjects included in this study, three subjects presented with episodes of respiratory attacks, which were confirmed by a pulmonologist to be symptoms of FLD. These subjects had a previous history and confirmed diagnosis of FLD (exposure to moulds, respiratory symptoms suggestive of the diagnosis, inspiratory crackles, low CO diffusing capacity, suggestive high resolution thoracic computed tomography scan features and lymphocytic proliferation observed in bronchoalveolar lavage). Serological tests performed at the end of the indoor feeding season were positive for the three patients. In two of these cases, we were able to identify the bales of hay which were at the root of contamination; the concentration of *A corymbifera* was very high (45 000 cfu/g) in one case and exceptionally high (110 000 cfu/g) in the other. Although the concentration of *E amstelodami* was also very high in these samples, it was not that exceptional. The presence of these microorganisms may be indicative of the risk for a given agricultural practice or it may be associated to other more pathogenic microorganisms. Nevertheless, the presence of seric precipitins shows that the farmers who experienced relapses had an immunological reaction against these antigens. These data confirm the results of our previous study,[7] by means of a different methodology, and provide new arguments for considering *A corymbifera* and possibly *E amstelodami* as causative agents of FLD. In the former study, in which FLD patients were matched and compared with healthy controls, we showed that the farms of patients suffering from FLD contained more *A corymbifera* than those of healthy farmers, and electrosyneresis, performed with *A corymbifera* and *E amstelodami* antigens, showed more arcs of precipitins in FLD patients than in control subjects. *W sebi* has also been suspected, but to a lesser degree than the other two microorganisms. Our current results argue in favour of exonerating *W sebi*.

Finally, even if these two studies do provide strong arguments for attributing FLD attacks to high levels of exposure to mould, the role of susceptibility and the effect of long term exposure cannot be excluded.[22] For these reasons, it is difficult to establish a level of exposure which correlates perfectly with the symptoms of FLD.

From a practical standpoint, classical measures such as the use of protective masks are seldom accepted, not even by patients who experience iterative relapses due to persistent exposure. Consequently, some patients stop working in order to preserve their respiratory function.[23] The fact that the proliferation of *A corymbifera* specifically concerns only some batches of hay, harvested in poor conditions, and that the most contaminated hay continues to deteriorate during storage may be of interest in the framework of implementing a prevention programme for patients with a history of FLD.

In conclusion, this study allows better characterisation of the circumstances which lead to the proliferation of FLD antigens in hay. It also strongly suggests a close relation between the concentration of moulds—especially *A corymbifera*—and the risk of occurrence of FLD. Our results open pathways for the implementation of preventive strategies for FLD patients.

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