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A 4-year study of the mycological aspects of Kashin-Beck disease in Tibet

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Abstract In order to clarify the association between mycotoxin-producing fungi in food and Kashin-Beck disease (KBD), we examined the occurrence and contamination levels of fungi in samples of barley grain, from KBD-affected families and from unaffected families in endemic areas. A control area without the occurrence of KBD served as reference. The first results obtained in 1995 showed that total mesophilic fungal contamination of barley grain was consistently higher in families with KBD. *Trichothecium roseum* (Pers) Link ex gray, *Dreschlera* Ito and *Alternaria* Nees ex Fr. were the three most common fungi significantly associated with KBD. In 1996 we again observed a significant difference between affected and non-affected families, especially with *Trichothecium roseum* and *Ulocladium* Preuss. On this basis, measures to prevent KBD were suggested and a preventive program has been set up since 1998 in 20 new villages.

Résumé Afin de vérifier l'hypothèse d'une relation entre la contamination des aliments par des moisissures et leurs toxines, avec la maladie de Kashin-Beck, nous avons évalué l'occurrence et le taux de contamination fongique dans les grains d'orge. Les études ont été menées en zones endémiques, chez des familles affectées par la maladie et chez des familles non-affectées. Une vallée contrôle, non affectée, a servi de référence. Les premières données obtenues en 1995 montre que le taux de contamination totale des grains d'orge par les moisissures mésophiles est régulièrement plus élevé chez les familles avec KBD. *Trichothecium roseum* (Pers.) Link ex gray, *Dreschlera* Ito et *Alternaria* Nees ex Fr. sont les trois fongus dont la présence est significativement corrélée avec la maladie. En 1996, nous avons à nouveau obtenu une différence significative entre les familles affectées et non affectées, particulièrement pour *Trichothecium roseum* et *Ulocladium* Preuss. Sur cette base, des mesures pour éviter le KBD furent suggérées et un programme préventif a démarré en 1998 dans 20 nouveaux villages.

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Introduction

Based on epidemiological investigations, the three main hypotheses for the aetiology of Kashin-Beck's disease (KBD) are considered to be selenium deficiency in food, a high concentration of organic matter (fulvic acid) in drinking water and severe contamination of food by fungal mycotoxins [2, 7, 10, 8, 15, 16, 17].

The possible role of mycotoxins in KBD was originally suggested by Russian researchers. Cereal grain contamination by *Fusarium sporotrichella* in endemic areas was mentioned by Nesterov in 1964 [16], and in China microbiological examinations have shown that wheat crops in KBD areas were contaminated by *Alternaria* sp. [2] and *Fusarium* spp. [12]. The Fusarium species, particularly *F. oxysporum* and *F. monoliforme*, were also isolated from corn [17]. Moreover, it was suggested that other fungi should also be considered. Mycotoxin contamination of corn and wheat were also investigated in KBD areas, and cereal samples in high incidence areas of KBD were reported to be more heavily contaminated with trichotecenes (T2-toxins) when compared to those in low incidence areas. Although Fusarium species are known to produce trichotecenes [22], these toxins are also formed by other species of cereal fungi such as *Trichothecium roseum* [8, 11], *Stachybotrys atra* [8] and *Myrothecium roridum* [8].

In order to clarify the association of fungi-producing mycotoxins with Kashin-Beck disease, we attempted to survey the occurrence and contamination levels of fungi in barley grain samples in KBD-affected families and KBD-non-affected families in Tibetan farmland in the Tibet Autonomous Region of the People's Republic of China.

Materials and methods

In 1995 two surveys were conducted in 12 villages of four counties of Lhasa Prefecture in the Tibet Autonomous Region (Fig. 1). In 1966 new villages were examined in Lhoca Prefecture and in Shigatse Prefecture, and these included a control valley (Rimpung County) where no KBD was found.

In 1995 the extent of KBD was assessed by the clinical examination of children aged between 5 and 15 years. In 1996 this age group was extended to those aged between 3 and 20 years in order to increase the size of the sample.

A case of KBD was defined as a child living in a KBD endemic area presenting with at least one chronic deformity of ankles, knees, interphalangeal joints, wrists or elbows and in the absence of either local inflammation or any history of trauma [13].

Observation and interview teams accompanied by a village official visited the homes of the children. The types of cereal grain, the total crop surface, grain storage containers and other parameters involving conditions within the houses were noted and recorded. Barley grain samples were checked for moisture content from each family home and the moisture content was measured using a programmable electronic moisture meter, "Samap HST 3".

In October 1995 during the harvest period, 60 samples of barley grain and 47 flours were collected from both KBD-affected and non-affected families of the 12 villages visited in Lhasa Prefecture. In November 1996, 36 new affected families and 36 without the disease were selected in other villages in the endemic areas of Lhasa, Lhoca and Shigatse prefectures. Thirty-six families in a control area (Rimpung Valley) without KBD were added to this study. All the grain samples were stored at –20°C until analysis in order to avoid any contamination developing in the storage bags.

A direct plating method [18] was used in order to determine the percentage of contaminated grains. After disinfection with an appropriate chlorine solution, grains of each sample were put into the agar medium with chloramphenicol: 50 grains into a malt agar to isolate mesophilic species, and 50 grains into an MY50S to isolate strongly xerophilic species. Plates were incubated at 25°C and the colonies counted after 5 days. The mould genus was identified, and the most interesting strains were stored for further identification and studies [13]. A dilution plating method [18] was used for flour samples. After incubation at 25°C the first examination and counting was done after 5 days, and this was repeated after 15 days. In October 1995 two agar media with chloramphenicol were used – MEA for mesophilic species and MY50S for xerophilic species [1].

Data were analysed using Stata 5.0 software. For bi-variate analysis chi-square, Student's *t*-test or Anova were used. Fischer exact test and the Kruskal-Wallis Anova were used when appropriate, and a multi-variate analysis was made with logistic regression. *P*-values of less than 0.05 were considered as significant.

Fig. 1 Prefectures in Tibet Autonomous Region

Results

Humidity and temperature were considered among the important parameters in this mycological study. Climatic conditions in Tibet are very severe. Precipitations generally fall from May to September with an annual total rainfall of between 263 mm and 563 mm in the South. In the North there is less rain. Average temperatures vary between –8.2°C in the North and +9.8°C in the Lhasa Region. Barley was the basic crop in all the villages, and was stored as grain or flour by 97% of the families studied. In the regions studied this cereal is planted in mid-April or May and harvested in September or October. The barley is then kept in bundles for 20 days in the fields and under these conditions the grain dries in the fresh air. Grains separated by threshing is then immediately stored in bags. Some barley is stored at home in different sorts of containers such as wool yak bags or large baskets. Plastic bags are also commonly used these days. The bags of grain, often weighing about 50 kg, are put in a storage room and generally lie directly on the ground.

While grain humidity 6 months after storage was within the normal range (median: 12.7%), grain humidity in October (recently harvested) was high (median: 17.5%) and this is too high on microbiological grounds. Moreover grain humidity was higher in KBD-affected families (18.3%) than in healthy families (17.4%).

Barley flour samples did not present a significant mould contamination difference between families with or without KBD. Neither was there any relation between the presence of bacteria and xerophilic species on grain, and the families affected. However, on the contrary in 1995 we noted a significant difference ($P<0.05$) between mesophilic moulds contamination of grains in the families with or without KBD [5]. In 1996, although the overall contamination rate was higher in endemic areas than during the previous year, we again observed a significant difference. This was more pronounced when genus Cladosporium (which may be considered as an important background) was removed. Another important observation was the low mould contamination of barley grain in the valley (56% and 42%, respectively, with and without Cladosporium; Table 1).

In respect of specific taxonomic groups (Table 2) the results obtained during 1995 in endemic areas showed three taxa which were significantly more abundant on grain sampled in families with KBD cases [4, 5] and

Table 1 Median values of contaminated stored grains percentages in different areas (*KBD+* families affected with the disease, *KBD–* non-affected families)

	Endemic area			Endemic area			Control	
	1995 KBD+ (1)	1995 KBD– (2)	*P*1/2	1996 KBD+ (3)	1996 KBD– (4)	*P*3/4	1996 KBD– (5)	*P*3/5
Median (tot. cont.; %)	66	43	<0.05	100	95	0.007	56	<0.0001
Median (without grains with *Cladosporium;* %)[a]	54	37&33[b]	0.01	89	69	0.0001	42	<0.0001
n families	30	30&28[b]		36	36		36	

[a] Calculated without grains contaminated by *Cladosporium* only
[b] Without two samples which presented an abnormal contamination

Table 2 Relation between genus fungal contamination found on barley grain and families affected or not affected with KBD (KBD diagnosis was in 1995 based on clinical examination of 5- to 15-year-old children, in 1996 on clinical examination of individuals 3–20 years old)

n families	October 1995					November 1996								
	Endemic areas					Non-endemic		Endemic areas				*P* ac	*P* bc	*P* ab
	Unaffected		Affected		*P*-value	Control (a)		Unaffected (b)		Affected (c)				
	30	%	30	%		36	%	36	%	36	%			
Acremonium	4	13.3	6	20.0	n.s.	3	8.3	5	13.9	7	19.4	n.s.	n.s.	n.s.
Acremoniella	3	10.0	6	20.0	n.s.	0	0	2	5.6	3	8.3	n.s.	n.s.	n.s.
Alternaria	8	26.7	19	63.3	0.004	1	2.8	25	69.4	31	86.1	<0.001	n.s.	<0.001
Arthrinium	6	20.0	10	33.3	n.s.	29	80.6	20	55.6	23	63.9	n.s.	n.s.	0.023
Aspergillus[a]	2	6.7	3	10.0	n.s.	6	16.7	8	22.2	14	38.9	n.s.	n.s.	n.s.
Aureobasidium	10	33.3	13	43.3	n.s.	1	2.8	2	5.6	2	5.6	n.s.	n.s.	n.s.
Chaetomium	5	16.7	10	33.3	n.s.	17	47.2	4	11.1	2	5.6	<0.001	n.s.	<0.001
Cladosporium	25	83.3	28	93.3	n.s.	36	100	36	100.0	36	100.0	n.s.	n.s.	n.s.
Cladosporium*	3	10.0	6	20.0	n.s.	15	41.7	31	86.1	32	88.9	<0.001	n.s.	n.s.
Drechslera	5	16.7	17	56.7	0.001	9	25.0	13	36.1	19	52.8	0.016	n.s.	n.s.
Fusarium	4	13.3	5	16.7	n.s.	7	19.4	22	61.1	15	41.7	0.041	n.s.	0.001
Humicola	1	3.3	2	6.7	n.s.	2	5.6	0	0	0	0	n.s.	–	n.s.
Monilia	0	0	0	0	–	0	0	0	0	2	5.6	n.s.	n.s.	–
Mucor	1	3.3	5	16.7	n.s.	2	5.6	3	8.3	4	11.1	n.s.	n.s.	n.s.
Nigrospora	0	0	0	0	–	0	0	10	27.8	12	33.3	<0.001	n.s.	0.001
Papulaspora	0	0	0	0	–	0	0	1	2.8	1	2.8	n.s.	n.s.	n.s.
Penicillium[a]	17	56.7	13	43.3	n.s.	16	44.4	14	38.9	20	55.6	n.s.	n.s.	n.s.
Phoma	9	30.0	16	53.3	n.s.	29	80.6	31	86.1	31	86.1	n.s.	n.s.	n.s.
Rhizopus	0	0	3	10.0	n.s.	10	27.8	9	25.0	8	22.2	n.s.	n.s.	n.s.
Scopulariopsis	0	0	0	0	–	2	5.6	0	0	1	2.8	n.s.	n.s.	n.s.
Stachybotrys	0	0	0	0	–	1	2.8	0	0	0	0	n.s.	n.s.	n.s.
Trichothecium	0	0	10	33.3	0.001	0	0	8	22.2	18	50.0	<0.001	0.014	0.003
Ulocladium	14	46.7	15	50.0	n.s.	5	13.9	8	22.2	26	72.2	<0.001	0.053	0.001
Ustilago	–	–	–	–	–	24	66.7	20	55.6	23	63.9	n.s.	n.s.	n.s.
Total yeasts	16	53.3	16	53.3	n.s.	32	88.9	30	83.3	34	94.4	n.s.	n.s.	n.s.
Sterile myc.*	14	46.7	21	70.0	n.s.	10	27.8	23	63.9	26	72.2	<0.001	n.s.	0.002
Unidentified	19	63.3	23	76.7	n.s.	30	83.3	31	86.1	31	86.1	n.s.	n.s.	n.s.
Mesophiles*	13	43.3	21	70.0	n.s.	21	58.3	34	94.4	36	100.0	<0.001	n.s.	0.001

[a] Genus Penicillium and *Aspergillus* are generally considered as post harvested contaminants
*≥10%

these were *Trichothecium roseum* (Pers) Link ex gray, *Dreschlera* Ito and *Alternaria* Need ex Fr. In 1996 differences between affected families were limited to *Trichothecium roseum* (P=0.014), *Alternaria* sp. and *Ulocladium* sp. (borderline significants P=0.089 and 0.053, respectively). But total contamination was higher everywhere because of a very rainy year. However, despite these very wet climatic conditions, control samples examined in the non-endemic areas contained up to eight taxa among which Alternaria, Trichothecium, Dreschlera, Ulocladium or Fusarium were found to be significantly less present than in endemic areas.

Discussion

Among fungal species, which correlated strongly with KBD, *Alternaria* is a very ubiquitous fungus, generally well represented by *A. alternata* gr. In cereal crops leaves and grains are colonised soon after appearance (sub-epidermal penetration). With minimal water activities for growth of 0.85–0.88 this species is considered as a "field fungi". Its pathogenicity is weak but it has been implicated in the conspicuous black or brown discoloration of cereal grains known as black point and it causes subsequent discoloration of flour. It is known to compete spatially with other species such as *Cladosporium, Epicoccum* and *Fusarium*. The large quantities of grains contaminated by this genus and by *Cladosporium* in our samples could explain the weak presence of Fusarium in 1995. Nevertheless there was a lot of sterile mycelium in which some parasite moulds such as *Fusarium* could be present (pink sterile mycelium for instance), but analyses of grain incubated on a specific medium for *Fusarium*, with malachite green [3], did not reveal more colonies than on malt agar. After being harvested *Alternaria* can survive in cereals and a high humidity (more than 20% for *A. alternata*) favours rapid colonisation of grains. In Tibet this situation occurs just after the harvest when barley is kept in bundles on the fields. This method is used for drying grain but is not always efficient because of contact with the wet soil and also because of the frequent presence of dew in some valleys. Fungal development can also occur rapidly when grain is stored in bad humidity conditions. Species of *Alternaria* are known to produce at least 70 secondary metabolites and many observations have pointed out their toxicity [6]. Isolated from over-wintered Russian cereal and supposedly involved in alimentary toxic Aleukia, *Alternaria alternata* is also reported to be the predominant fungus isolated from wheat seeds in the Tianshui area of Gansu Province (China) where Kashin-Beck disease is prevalent [2]. Analysis of the metabolic extract shows the presence of alternariol, methyl ether and tenuzonic acid, which added to the diet of rats produces a significant decrease in Se-GSH-Px and SOD activities, and an acceleration in lipid peroxidation [2]. These results point in the same direction as the free radicals hypothesis of KBD [20] and prove that supplement of selenium is effective in preventing the toxicity of *Alternaria* extract. Another interesting related fact was given by Thompson-Eagle et al. [19] who noted that *Alternaria alternata* when isolated as an active Se-methylating organism produces a volatile compound identified as dimethylselenide. We assume that this property could be important in areas already deficient in selenium especially if contamination of grain (in the fields or in storage) by *Alternaria* was able to produce an additional leak of selenium.

Cladosporium has a similar ecology as *Alternaria* and is found to be widespread on the ears of cereals at harvest (lemma, outer tissues of the kernels such as hull and pericarp). Its ability to thrive a low temperatures allows it to grow on chilled and over-wintered grain, and these conditions exist in Tibet. *Trichothecium roseum*, the third main isolated species, also has a worldwide distribution and is commonly found on decaying plant substrata and in cereals which are stored in bad conditions. With a minimal water activity for growth of 0.90, this saprophytic species is classified as a post harvest contaminant especially in the early stage of storage when grains are kept in too high humidity. Numerous mycotoxins produced by these taxa are known as trichothecin and belong to the group of trichothecenes which are implicated in some bone pathologies [8, 20, 21]. *Dreschlera* Ito 1930 (belonging to the *Helminthosporium*) is a genus with many parasitic species. *H. gramineum, H. teres* and *H. sativum* are generally found on barley and their primary infection of plants is usually generated from seeds. This infection process is favoured by all factors tending to prolong the duration of germination and thus increase the expression of illness. In Tibet low temperatures, or soil characteristics which can vary from valley to valley, could explain the relative proximity of endemic areas and healthy areas. In addition mycelium present in seeds may remain viable for several years, but at the present time no human or animal pathogenicity has been noted.

In 1996 there was more rain in Tibet than in 1995, and global fungal contamination was higher everywhere. However, the different rates of contamination between KBD families and healthy families in endemic areas, and especially between endemic areas and the control area, were maintained.

It should be stressed that grain is generally stored at home and that airborne fungal spores may be more dangerous for health when inhaled. For example, the toxicity of inhaled trichothecenes is considered to be 40 times higher than ingested trichothecenes.

In conclusion the agricultural and storage conditions of home-produced grain in Tibet are far from optimal. Fungal contamination of barley grain and especially the presence of several taxa such as *Trichothecium roseum, Dreschlera* and *Alternaria*, are significantly related to the presence of KBD in families while a control valley with no KBD cases showed a very low level of fungal contamination of grain. In 1996 up to eight taxa including *Alternaria, Trichothecium, Dreschlera, Ulocladium* or *Fusarium* were found to be significantly less present in the control valley than in the endemic area. Annual variations of climatic conditions which induced more or less fungal contaminations did not alter this difference.

Our findings suggest that in Tibet during the three critical periods of microbiological barley contamination fungi related to KBD are commonly present: firstly during germination by contaminated seed (*Dreschlera*); secondly in the fields, especially in summer when some parasitic moulds (*Alternaria, Cladosporium, Dreschlera*) may invade the ears of grain; and thirdly at the beginning of storage when drying has been inadequate (*Trichothecium, Alternaria, Cladosporium*).

The intermediate period just after the harvest and before storage when the barley is kept in bundles in the fields also requires further study.

As a result of our study a health improvement programme was started in 1997 in 20 villages and this will continue until 2001. Appropriate measures to prevent KBD in Tibet have been taken and fungal analyses are made every year to assess the effect of these actions.

The programme includes:

- Disinfection of seeds before planting, or the use of new healthy grains for new areas of cultivation. Fungicide spraying (Azoxistrobine) when the seed-bearing heads of the barley start to form.
- Improving drying methods for grain before storage with one person per village being put in charge of controlling grain humidity before storage (using a Samap device). Storage is acceptable when the humidity level is below 14%.
- Improving storage conditions. Cleaning, whitewashing, replacing old bags and containers with new plastic bags in the first 2 years and avoiding contact of bags with wet ground.
- Informing the population of the risks associated with eating mouldy grains, and of the importance of storing grain in good conditions. Keeping everyone informed about the health risks of different chemical products such as those used for treating seeds, and for their use during fungicide preparation and spraying.
- Establishing control units similar to the mycology laboratory in Lhasa, which was opened in 1998.

References

1. Atlas RM (1993) Handbook of microbiological media. Parks (ed)
2. Bai FY, Chen YD, Chen QT (1990) Investigation on the contamination fungi of grain in Tian-Shui Kashin-Beck disease areas. Chin J Cont Endem Dis 5:33–34
3. Castella G, Bragulat MR, Rubiales MV, Cabanes FJ (1997) Malachite green: a new selective medium for *Fusarium* spp. Mycopathologia 137:173–178
4. Chasseur C, Suetens C, Haubruge E, Mathieu F, Begaux F, Tenzin T, Nolard N (1996) Grain and flour storage conditions in rural Tibetan villages affected by Kashin-Beck disease in Lhasa Prefecture, Tibetan Autonomous Region: environmental approach. In: PA Miller (ed) Proceedings of the International Congress of Bacteriology and Applied Microbiology, Jerusalem
5. Chasseur C, Suetens C, Nolard N, Begaux F, Haubruge E (1997) Fungal contamination in barley and Kashin-Beck disease in Tibet. Lancet 350:1074
6. Chelkowski et al. (1991) Alternaria and their metabolites in cereal grain. In: Cereal grain. Elsevier, pp 607
7. Doyle MP, Beuchat LR, Montville TJ (1997) Food microbiology: fundamentals and frontiers. ASM Press, Washington, pp 768
8. Girisham S, Rao GV, Reddy SM (1985) Mycotoxin producing fungi associated pearl millet. Natl Acad Sci Lett 8:333–335
9. Ishii K, Kobayashi J, Ueno J, Ichinoe M (1986) Occurrence of trichotecin in wheat. Appl Environ Microbiol 52:331–333
10. Jiang YF, Xu GL (1989) The relativity between some epidemiological characteristics of Kashin-Beck disease and selenium deficiency. In: Wendel A (ed) Selenium in biology and medicine, Springer, Berlin Heidelberg New York, pp 179–183
11. Krogh P et al. (1989) Natural occurrence of the mycotoxin fusarochromanone, a metabolite of *Fusarium equiseti*, in cereal feed associated with tibial dyschondroplasia. Appl Environ Microbiol 55:3184–3188
12. Luo Y, Yoshizawa T, Yang J-S, Zhang S-Y, Zhang B-J (1992) A survey of occurrence of Fusarium mycotoxins in corn and wheet samples from Shanxi and Shanxi Provinces, China. Mycotoxin Res 8:85–91
13. Mathieu F, Begaux F, Zhang Yong Lan, Suetens C, Hinsenkamp M (1997) Clinical manifestations of Kashin-Beck disease in Nyemo Valley, Tibet. Int Orthop 21:151–156
14. Mathre DE (1997) Compendium of barley disease. APS Press, pp 90
15. Moreno-Reyes R et al (1998) Kashin-Beck osteoarthropathy in rural Tibet in relation to selenium and iodine status. N Engl J Med 3339:1112–1120
16. Nesterov I (1964) The clinical course of Kashin-Beck disease. Arthritis Rheum 7:29–40
17. Peng A, Yang C, Rui H, Li H (1992) Study on pathogenic factors of Kashin-Beck disease. J Toxicol Environ Health 35: 79–90
18. Pitt JI, Hocking AD, Samson RA, King AD (1992) Recommended methods for mycological examination of foods. In: Mod Methods Food Mycol 31:365–375
19. Thompson-Eagle ET, Frankenberger JR, Karlson U (1989) Volatilization of selenium by *Alternaria alternata*. Appl Environ Microbiol 55:1406–1413
20. Wang K et al (1991) Free radicals induced abnormal chondrocytes, matrix and mineralisation. Chin Med J 104:307–312
21. Yang JB (1983) Progress in studies on effect of Fusarium toxin on Kashin-Beck disease. Chin J Endem Dis 3:155–161
22. Yongmin Xiong (1997) Toxic effect of T-2 toxin on articular cartilage in Chinese experimental mini-pig. Chin J Endem Dis 12:4–6