



Protein Profiles of *Aspergillus* Species Isolated From the Tea Gardens and Factories Air in Northern Iran

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ABSTRACT

Background: Spoilage of tea (*Camellia sinensis*) is often accompanied by contamination and formation of mycotoxins, which are toxic secondary metabolites produced by specific tea-borne fungi.

Objectives: The purposes of this study were to isolate *Aspergillus* flora in the air from tea gardens and factories in Gilan and Mazandaran provinces, in northern Iran, and to determine their protein patterns.

Materials and Methods: Air samples were collected from 11 tea gardens and 13 tea factories between 2006 and 2008, and cultured in specific fungal media. Mycelial mats and spores of the *Aspergillus* species were disrupted in liquid nitrogen containing glass beads. The crude extracts were separated from other cell components by centrifugation and sterilized using a filter. The extracts obtained were analysed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to separate the fungal antigenic proteins.

Results: A total of 4 157 fungal colonies were observed in the tea gardens air and 3 950 colonies in the tea factories air. The most dominant and frequently occurring *Aspergillus* flora were; *A. niveus* (14.4%) and *A. unguis* (12.6%) in the gardens, and *A. melleus* (14.3%) and *A. niger* (12.3%) in the factories. The highest number of *Aspergillus* species collected from the tea gardens and factories were associated with the regions of Lahijan (23.9%) and Rasht (18.8%), respectively. The results from the SDS-PAGE method indicated that various *Aspergillus* species had 55 protein bands, which ranged from 5 to 120 kDa. Among these species, *A. foetidus* had the maximum number of protein bands (22 bands) ranging from 14.4 to 120 kDa, while *A. unguis* contained the minimum protein bands (5 bands) ranging from 30 to 120 kDa.

Conclusions: The results revealed the presence of a variety of *Aspergillus* species in tea gardens and factories air, and the presence of a large number of *Aspergillus* species based upon the protein banding patterns obtained with SDS-PAGE analysis.

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► Implication for health policy/practice/research/medical education:

Whole-cell protein profiles of *Aspergillus* species could provide valuable criteria for serological and immunological studies of tea workers exposed to occupational aeroallergens.

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1. Background

Aspergillus species are a common group of filamentous fungi, which have universal dissemination and are readily recovered from; soil, decaying vegetation, air and many other environments (1). They produce a large number of conidia (spores), which are turned into aerosols and distributed in the environment. Because of their small size (< 5 µm) and the aerodynamic properties of the *Aspergillus* conidia, they can pass through the upper respiratory tract defenses and may reach distal regions of the lungs (2). *Aspergillus* is a mould that is responsible for a gamut of respiratory diseases ranging from; saprobic colonization to rapidly invasive disseminated disease, especially in individuals with weakened immune systems (3). *Aspergillus* species are an important source of allergen proteins and have been shown to produce 90 different allergens (4). The clinical spectrum of *Aspergillus* associated hypersensitivity respiratory disorders includes; asthma, allergic bronchopulmonary aspergillosis (ABPA), allergic *Aspergillus* sinusitis (AAS) and hypersensitivity pneumonitis. Although *A. fumigatus* is the most common *Aspergillus* species known to trigger allergic disorders; *A. niger*, *A. flavus*, *A. nidulans*, *A. oryzae* and *A. terreus* have also occasionally been responsible for these conditions (5).

Tea (*Camellia sinensis*) is one of the most common drinks in the world, enjoyed by many people chiefly in the Far East, Middle East, Europe, Australia and Africa. It has been extensively cultivated in the north of Iran since ancient times and it is known to carry various mycoflora just like any other crop plants, some of which may secrete toxins. Previous studies have shown that the most common fungi isolated from tea gardens are; *A. flavus* and other species of *Aspergillus* and *Penicillium* (6). The processing of black tea involves a series of stages including; withering, rolling, fermenting and firing, before the final product is completed (7). Fungi have been isolated from all stages of tea processing and manufacturing, and recontamination of the final product after firing may also occur during sorting and packaging (8). The possibility that tea may serve as a vehicle for human pathogens has been reported in earlier studies (9). However, no systematic study has been carried out concerning the air spora of tea gardens in Iran, up to now. The high average temperatures and relative humidity of the Gilan and Mazandaran provinces, which are accompanied by poor conditions and prolonged duration of tea storage, promotes the growth of different fungi, in particular *Aspergillus* species.

2. Objectives

The purposes of this study were to isolate *Aspergillus* flora from tea gardens and factories air in certain regions of Gilan and Mazandaran provinces in northern Iran and to determine the protein patterns of various *Aspergillus* isolates.

3. Materials and Methods

3.1. Isolation and Identification of *Aspergillus* Species

Thirteen regions of Gilan and Mazandaran provinces were selected for data collection; Rasht, Lahijan, Amlash, Rudsar, Foman, Siahkal, Shaft, Langerud, Ramsar, Tonekabon, Chalus, Somesara and Astaneh. The samples were collected using five petri dishes including malt extract agar (MEA) (Merck Co., Darmstadt, Germany), yeast extract agar (YEA) (Merck Co., Darmstadt, Germany), Czapek Dox agar (CDA) (Sigma, St Louis MO, USA), Czapek yeast extract agar (CYEA) (Sigma, St Louis MO, USA) and Sabouraud dextrose agar (SDA) (Merck Co., Darmstadt, Germany) containing chloramphenicol (100 ppm) and tetracycline (50 ppm), from May to October of 2006 to 2008 (10). With the exception of rainy days, samples were exposed to the air for 30 min at a height of 1.5 m above the ground. After five days incubation at 25°C, the colonies were isolated in the various media and observed for the formation of macrocolonies. Micromorphology was observed after microcultivation and *Aspergillus* strains were identified using the classifications of Klich (11), Kojakovic (12) and Samson (13).

3.2. Cell Fractionation and Crude Extract Preparation

Each fungal isolate was cultured in Czapek yeast extract agar at a temperature of 25°C for 5 days and then incubated in Czapek Dox broth medium. Incubated broth media were kept at 25°C for 7 days on a gyratory shaker (200 rpm). Fungal mycelia and spores were separated from the broth media by centrifugation at 3 000 g for 15 min. The pellets were washed 3 times with 10 mM Phosphate buffered saline (PBS), pH 7.5 by centrifugation at 3 000 g for 15 min. The fungal elements were disrupted in liquid nitrogen containing glass beads (diameter; 1 mm) for 25 min. After cell disruption, the crude extracts were separated from other cell components by centrifugation at 3 000 g for 15 min. The extracts were sterilized using a filter (0.2 µ, Sartorius, USA) and stored at -20°C until required.

3.3. Determination of Protein

Protein concentration of the sample was determined after reconstituting in 50 and 100 µl of distilled water by the Bradford method (14).

3.4. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed using the Laemmli method (15). The fungal extracts were dissolved in a sample buffer (Novex, San Diego, CA, USA) and boiled for 5 min. Aliquots of the sample (volum = 10 µl, which equals 60 µg of the protein) and standard markers (3 ± 185 kDa) (Novex, San Diego, CA, USA) were applied to a Novex precast NuBis-Tris gel (4 ± 12%) for the separation of fungal antigenic proteins. Electrophoresis was performed with a Novex X

cell II mini-cell for 40 min at a constant voltage of 200 watt. The gel was fixed and stained with 0.1% Coomassie Brilliant Blue.

3.5. Statistical Analysis

The data analysis was undertaken with a SPSS (version no. 12) program using the Student's t-test statistical method for analysis of significance. A *p* value less than 0.05 was regarded as significant.

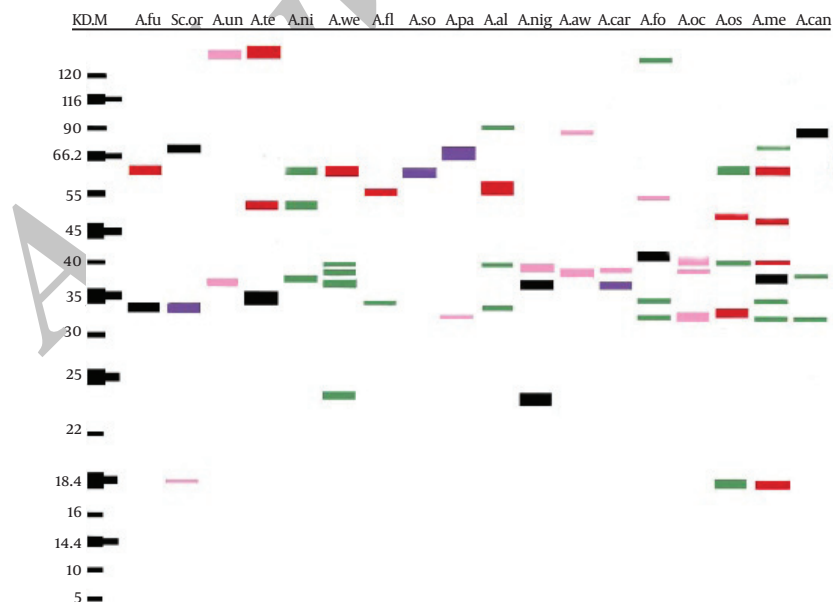
4. Results

The results of the aeromycoflora study showed that the air of tea gardens and factories is never free of fungal spores. The sum of 4 157 (51.3%) and 3 950 (48.7%) *Aspergillus* colonies were obtained from the tea gardens and factories of Gilan and Mazandaran provinces, respectively. Of those colonies, 16 species were identified from gardens and 14 species from factories. As illustrated in Table 1, *A. niveus* (14.4%), *A. unguis* (12.6%) and *A. alliaceus* (9.7%) were the most frequent *Aspergillus* species isolated from the tea gardens, while *A. melleus* (14.3%), *A. niger* (12.3%) and *A. flavus* (11.6%) were found to be the most predominant *Aspergillus* species obtained from the tea factories. Our results showed that toxigenic species such as; *A. flavus* and *A. parasiticus*, were higher in number than the other *Aspergillus* species, especially inside the tea factories. Table 1 shows the population of different *Aspergillus* species from 11 tea gardens and 13

tea factories in a number of Iranian regions. The highest number of *Aspergillus* species that were obtained from tea gardens were associated with the regions of Lahijan (23.9%), followed by Rudсар (14.7%) and Rasht (14.3%). The tea factories with the highest level of *Aspergillus* species contamination were found in Rasht (18.8%), followed by Lahijan (16.4%) and Rudсар (11.8%).

In the present study, results from the SDS-PAGE method indicated that extracts obtained from various *Aspergillus* species had 55 protein bands, which ranged from 5 to 120 kDa (Figure 1 and Table 2). Most bands were concentrated between 14.4 and 55 kDa in all of the species. Among these species, *A. foetidus* had the maximum number of protein bands (22 bands) ranging from 14.4 to 120 kDa and *A. unguis* had the minimum protein bands (5 bands) ranging from 30 to 120 kDa. Among the different bands, protein bands with a molecular weight of 120 kDa were present in all species of *Aspergillus* except for; *A. fumigatus*, *A. parasiticus*, *A. carbonarius*, *A. ochraceus* and *A. ostianus*. A band with a weight of 116 kDa was observed in all of the 18 species of *Aspergillus* except for; *A. wentii*, *A. parasiticus* and *A. awamori*. Thus, common protein bands including 120 and 116 kDa were detected with a frequency of 72.3% and 83.4% in different species of *Aspergillus*, respectively. Regarding the results of common protein bands and according to the statistical analysis, no meaningful relationship was observed among the protein bands obtained from different *Aspergillus* species in this current study.

Figure 1. Electrophoretic Patterns of *Aspergillus* Species Isolates Taken from Tea Gardens and Factories Air.



Abbreviations: M, Marker; A.fu, *Aspergillus fumigatus*; Sc.or, *Sclerocleista ornata*; A.un, *Aspergillus unguis*; A.te, *Aspergillus terreus*; A.ni, *Aspergillus niveus*; A.we, *Aspergillus wentii*; A.fl, *Aspergillus flavus*; A.so, *Aspergillus sojae*; A.pa, *Aspergillus parasiticus*; A.al, *Aspergillus alliaceus*; A.nig, *Aspergillus niger*; A.aw, *Aspergillus awamori*; A.car, *Aspergillus carbonarius*; A.fo, *Aspergillus foetidus*; A.oc, *Aspergillus ochraceus*; A.os, *Aspergillus ostianus*; A.me, *Aspergillus melleus*; A.can, *Aspergillus candidus*

Table 1. Number of *Aspergillus* Species Isolated From Different Tea Gardens and Factories Air in Gilan and Mazandaran Provinces.

	Tea Gardens											Tea Factories													
	Rasht	Rudсар	Lahijan	Foman	Ramsar	Shaft	Langerud	Chalus	Amlash	Siahkal	Tonekabon	Rasht	Rudсар	Lahijan	Foman	Ramsar	Shaft	Langerud	Chalus	Amlash	Siahkal	Tonekabon	Somesara	Astaneh	
<i>A. fumigatus</i>	150	14	55	0	31	0	0	6	28	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Scierocleista ornata</i>	0	0	88	140	0	0	0	0	0	92	0	0	0	89	0	0	0	0	0	0	93	86	0	0	0
<i>A. unguis</i>	115	118	117	53	120	0	0	0	0	0	0	0	0	67	0	0	0	0	0	65	0	0	52	0	0
<i>A. terreus</i>	0	7	0	0	40	33	0	0	0	0	0	0	66	0	64	0	124	0	27	0	0	0	0	0	0
<i>A. niveus</i>	104	151	103	0	105	0	135	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>A. wentii</i>	0	0	100	0	0	0	101	0	0	0	0	128	0	0	0	0	0	0	102	0	0	0	0	0	0
<i>A. flavus</i>	68	36	0	61	22	0	34	0	0	16	9	147	0	80	0	0	137	0	11	17	0	0	63	4	0
<i>A. sojae</i>	0	18	41	5	0	19	0	0	0	0	0	69	0	0	0	0	0	0	0	0	1	0	0	30	0
<i>A. parasiticus</i>	0	0	123	0	139	95	0	0	0	0	0	96	0	0	94	0	43	0	45	0	0	0	0	0	0
<i>A. alliaceus</i>	0	0	0	85	131	0	54	0	0	132	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>A. niger</i>	0	138	84	0	0	0	0	0	0	0	0	78	0	0	0	129	0	47	153	0	0	0	79	0	0
<i>A. awamori</i>	126	0	76	0	0	0	0	0	0	0	0	0	49	0	0	0	42	0	0	0	0	0	0	0	0
<i>A. carbonarius</i>	0	0	75	0	0	3	0	0	0	0	0	71	74	0	0	0	0	0	0	0	0	0	0	0	0
<i>A. foetidus</i>	0	0	0	83	0	0	0	0	0	0	0	0	0	0	0	0	77	46	0	39	0	0	0	0	0
<i>A. ochraceus</i>	32	0	107	0	0	0	0	0	0	0	0	70	125	106	0	0	0	0	0	0	0	0	0	0	0
<i>A. ostianus</i>	0	130	26	37	0	0	0	0	0	136	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>A. melleus</i>	0	0	0	0	0	0	0	0	0	0	0	127	142	99	0	0	98	0	97	0	0	0	0	0	0
<i>A. candidus</i>	0	0	0	0	0	0	0	0	0	0	0	90	82	0	0	0	0	0	0	0	0	145	87	0	0
Total	595	612	995	464	443	262	67	141	183	108	287	743	466	646	158	253	312	205	260	268	93	231	281	34	0

Table 2. Number of Protein Bands Observed by the SDS-PAGE Technique in Each of the 18 *Aspergillus* Species Isolates.

	Protein bands, kDa	Total (bands)
<i>A. fumigatus</i>	116, 66.2, 45, 35, 30, 16.4	6
<i>Sclerocleista ornata</i>	120, 116, 110, 68, 60, 50, 45, 35, 26, 18.4, 16	11
<i>A. unguis</i>	120, 116, 55, 37, 30	5
<i>A. terreus</i>	120, 116, 66.2, 60, 55, 45, 35, 30, 27	9
<i>A. niveus</i>	120, 116, 70, 66, 55, 38	6
<i>A. wentii</i>	120, 66.2, 47, 45, 40, 37, 35, 28, 25, 24, 14.4	11
<i>A. flavus</i>	120, 116, 90, 66.2, 59, 55, 45, 44, 40, 35, 32, 27, 26, 22, 18.4, 17.6, 14.4	17
<i>A. sojae</i>	120, 116, 70, 66.2, 40, 30, 28, 14.4	8
<i>A. parasiticus</i>	116, 66.2, 70, 55, 50, 45, 42, 37, 33, 25, 17, 15, 14.4, 10, 5	15
<i>A. alliaceus</i>	120, 116, 92, 57, 47, 40, 35, 34, 30, 14.4	10
<i>A. niger</i>	120, 116, 75, 57, 45, 40, 37, 35, 32, 25, 24, 14.4	12
<i>A. awamori</i>	120, 90, 45, 40, 25, 24, 20, 18.4	8
<i>A. carbonarius</i>	116, 90, 66.2, 55, 54, 45, 43, 39, 37, 34, 33, 30, 27, 25, 23, 18.4, 17.4, 14.4, 10	19
<i>A. foetidus</i>	120, 116, 95, 90, 66.2, 55, 45, 44, 43, 42, 41, 37, 35, 33, 30, 25, 24, 20, 10, 18.4, 17.4, 14.4	22
<i>A. ochraceus</i>	116, 66.2, 47, 45, 44, 41, 40, 39, 34, 30, 29, 24, 20, 19.4, 18.4, 17.4, 15.4, 14.4, 10	19
<i>A. ostianus</i>	116, 66.2, 55, 44, 43, 41, 37, 32, 30, 27, 18.4, 16.4, 10	13
<i>A. melleus</i>	120, 116.2, 90, 80, 70, 66.2, 53, 46, 42, 40, 38, 35, 33, 30, 20.4, 18.4, 17, 14.4	18
<i>A. candidus</i>	120, 116, 90, 55, 45, 40, 38, 33, 26	9

5. Discussion

Aspergillus species are well-known as causative agents of opportunistic infections in humans. *Aspergillus* antigens can also induce the development of hypersensitive respiratory processes (4), although their frequency, epidemiological characteristics and clinical relevance are still unknown, this is partly due to insufficient knowledge of these antigens.

In this present study, a total of 4 157 and 3 950 *Aspergillus* colonies were obtained from tea gardens and factories, respectively, from different regions of Gilan and Mazandaran provinces. The most frequent *Aspergillus* species isolated from the tea gardens and factories were found to be *A. niveus* (14.4%) and *A. melleus* (14.3%), respectively. In a study conducted by Sharma (16), *A. fumigatus* had the highest percentage contribution (18%), followed by *A. flavus* (12.6%), *A. niger* (9.2%), *A. ochraceus* (2.3%) and *A. japonica* (2.3%) in the air of the Darjeeling tea garden, India. Hasan and Abdel-Sater (17) indicated that *Aspergillus* was the most predominant genus encountered in all of the black tea samples comprising 92.3% of the total fungi. From the *Aspergillus* genus, 11 species and 1 variety were identified of which; *A. flavus*, *A. fumigatus*, *A. niger* and *A. terreus* were the most common. Other studies have reported *Aspergillus* species to be the most dominant fungi in all of the tea brands (18, 19).

A few of the fungal isolates are known to be mycotoxin producers, such as; *A. flavus* and *A. parasiticus* (20). In this study, the occurrence of these toxigenic fungi was high in comparison to that of the other *Aspergillus* species, especially in the tea factories. Our results are not in agreement with Dutta *et al.* (21), who reported a low frequency of aflatoxigenic *Aspergillus* species in the air of a tea fac-

tory in the Cachar district, Assam, India. Although not all *A. flavus* strains are aflatoxigenic, a high incidence of aflatoxigenic strains (50-100%) are usually found among *A. flavus* isolates (22). Keeping the above view in mind, information regarding the probable mycotoxin producing air mycoflora in relation to the tea garden and factory and their mycotoxin producing ability in tea, it should be given due importance. Mycotoxins are secondary fungal metabolites with well-known health effects including; carcinogenesis, immunosuppression and cytotoxicity on alveolar macrophages (23). They are products that become poisonous and elicit a toxic response known as mycotoxicoses when food or feed containing them is eaten by humans or animals (24). There is evidence that aflatoxins can become air-borne and circumstantial evidence suggests that aflatoxins may also occur in the air. In fact, aflatoxins have been found in; spores, fungal hyphae and substrate materials, all of which could be released into the air when toxigenic fungi sporulate on indoor building materials. Their rate of inhalation can be up to 40 times greater than by ingestion (25). This raises the possibility of potentially hazardous exposures to the inhabitants of contaminated spaces.

In the present study, the highest number of *Aspergillus* species obtained from the tea gardens and factories were associated with the regions of Lahijan (23.9%) and Rasht (18.8%), respectively. Aerosolized *Aspergillus* spores are found nearly everywhere so we are routinely and almost constantly exposed to them. They can cause human and animal diseases through the production of mycotoxins, by the induction of allergenic responses and through localized or systemic infections (26). Occupational mycoses are forms of extrinsic allergic alveolitis. They are all

inflammatory reactions caused by breathing in high concentrations of fungal spores and other antigenic organic matter. Farmer's lung, one of the best known of these, is an occupational mycosis correlated with inhalation of high concentrations of *Aspergillus* spores from contaminated agricultural products (27).

The results from the SDS-PAGE analysis indicated that extracts obtained from various *Aspergillus* species contained 55 protein bands with molecular weights between 5 and 120 kDa. The highest protein bands were associated with *A. foetidus* (22 bands) in a range from 14.4 to 120 kDa, while the lowest ones were detected in *A. unguis* (5 bands) in a range from 30 to 120 kDa. Protein bands of 120 and 116 kDa had 72.3% and 83.4% frequencies in different species of *Aspergillus*, respectively. Our results are in close agreement with previous investigations. In a study by Saeednejad et al. (28), 69 protein bands with molecular weights between 11.5 and 178 kDa were identified. Among these, protein bands with molecular weights of 15, 23.5, 27, 33.5 and 61 kDa were found in two species, *A. fumigatus* and *A. flavus*, protein bands 28.5, 40 and 47 kDa were found in two species of *A. flavus* and *A. niger*, and a band with 120 kDa was found in *A. fumigatus* and *A. niger*. Identification of a protein in a complex antigenic extract on the sole basis of its molecular weight is a difficult process and sometimes the same antigen may have small differences in molecular weight in different studies. In addition, the differences observed in molecular weight may be related to differences in the calculation of the molecular weight or to the presence of several antigens with the same molecular weight, the species used as the source of antigenic proteins and sometimes even the methods of detection.

In conclusion, *A. niveus* and *A. melleus* were the predominant *Aspergillus* species found in tea gardens and factories air, respectively. In SDS-PAGE, 22 and 5 protein bands were detected in *A. foetidus* and *A. unguis*, respectively. The whole-cell proteins profile obtained by the SDS-PAGE analysis can provide valuable criteria for serological and immunological studies of tea workers exposed to occupational aeroallergens. Also, the results obtained do not support the concept that such protein patterns can be used in the identification of species, however, it suggests that this technique might be of value in assessing the degree of similarity among species of the same genus or of closely related genera.

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Authors' Contribution

None declared.

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