Presence of Aflatoxin-producing *Aspergillus* in Japan

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The outbreak of the Turkey "X" disease(ref. 1) in England in 1960 focused world attention to mycotoxins especially aflatoxins. After *Aspergillus flavus* was identified as the causative biological factor(ref. 2) for this disease, the contamination of aflatoxin in Japanese fermented foods was seriously considered because *A. oryzae*(Koji mold), a closely related fungus to *A. flavus*, is commonly used for the production of these foods. To test this possibility, more than two hundred *Aspergillus* that are used for food production in Japan were analyzed in our laboratory and we found that none of these fungi were aflatoxin producers(ref. 3). We then analyzed over one hundred different types of Japanese fermented foods that were either produced commercially or home-made. Again, we did not find any aflatoxins in these foods. In 1969, Dr. Kurata found(ref. 4) an aflatoxigenic fungus in a home-made soy paste in Japan. Because the location of food production may selectively affect the growth of the fungi, we surveyed soils and cereals from Hokkaido, the northernmost island of Japan, to Okinawa south of the mainland as well as some countries in southeast Asia for the toxigenic fungi. The results showed that aflatoxigenic fungi were present in very restricted areas in the southwest portion of the main and southern islands of Japan. The frequency of detection of the aflatoxigenic fungi increased in the samples collected in the south as compared to the north(ref. 5). Due to the presence of aflatoxigenic fungi on the silkworm, a well-known silkworm disease called "koji kabi disease" in Japan is thought to be caused by *Aspergillus* fungi. To determine the extent of fungal contamination in the silkworm population, we collected samples

from silkworm raising places throughout of Japan and analyzed for the *Aspergillus* fungi. In addition, the capability of these fungi for the production of aflatoxins and other mycotoxins was determined.

Materials and Methods

Modified malt agar medium was used for the isolation of fungi from samples. One liter of medium contained 45 g of malt agar, 30 g of sodium chloride, 30 mg of Chloramphenicol, 30 mg of Rose Vengal and 1 mg of Botran. The sample(1 g) was suspended in 0.01 % Tween 20 solution(100 ml), then mixed the medium in petri dishes and incubated at 27 °C in the dark. After 48 to 96 hours, the *A. flavus* group fungi were transferred individually to a standard malt agar medium in a test tube.

For aflatoxin production, all *A. flavus* group fungi isolated from samples were inoculated onto a rice medium containing 30 g of polished rice medium and 1 ppm of zinc and incubated at 27 °C for 7 days in the dark. After incubation, the cultures were autoclaved and kept at -20 °C until analysis. Aflatoxins were extracted from the cultures with chloroform and then subjected to a silica-gel alumina column for purification. The eluate containing aflatoxins were evaporated to dryness, dissolved in benzene-acetonitrile and then analyzed by normal phase HPLC with fluorescence detector(ref. 6,7).

Some isolates were tested for their ability to produce of cyclopiazonic acid(CA), one of the mycotoxins which was also produced by *A. flavus* group fungi using the methods previously described in ref. 8 and 9.

Results and Discussions

As shown in Table 1, *A. flavus* group fungi were isolated from 53 out of 92 dust and excrement samples collected at silkworm raising places and 41 of them produced aflatoxins. However, from 36 soil samples collected either close to silkworm raising places or mulberry fields where silkworm excrement and remnants of mulberry leaves(the only material feed to silkworms) were returned as manure, only 3 aflatoxin producing fungi and 8 *A.*

flavus group fungi were detected. The 3 fungi isolated from the soil samples were collected either in the silkworm raising place or just outside of it. It appears that these fungi were had difficulty in establishing in soil.

Table 1 *Aspergillus flavus* group fungi isolated from cocooneries in Japan

	Northern	Central	Southern	Total
No. of soil samples	19	10	7	36
No. of samples <i>A. flavus</i> isolated	4	3	1	8
No. of aflatoxigenic fungus	1*1	1*2	1*1	3
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No. of dust and excrements samples	32	14	46	92
No. of samples <i>A. flavus</i> isolated	15(47*3)	7(50)	31(67)	53(58)
No. of aflatoxigenic fungi	9(28)	5(38)	27(59)	41(46)

*1. Sample collected just outside of silkworm raising place

*2. Sample collected in a silkworm raising place

*3. percent of sample

The proportion of both *A. flavus* group fungi positive samples and aflatoxigenic fungi positive samples increased from north to south. This result is similar to our previous observations on the distribution of *A. flavus* in soil samples(ref. 5).

Twenty-six fungi isolated from different silkworm raising places were tested for their ability to produce CA. Seven of cyclopiazonic acid producers, either aflatoxigenic or nonaflatoxigenic were found(3.3-125 ppm). This proportion of CA producers was higher than in our previous study which we analyzed the production of CA by Japanese koji molds(ref. 9). We also analyzed 23 of *A. flavus* group fungi isolated from India and some southeast Asian countries for their ability to produce CA. Fifteen out of these 23 fungi produced CA(0.6-98 ppm). Again, the proportion of CA producers in this group is much higher than those isolated in Japan

Conclusion

Aspergillus flavus group fungi were not frequently isolated from soil in the northern to central part of Japan but were often isolated from silkworm excrement. Aflatoxigenic fungi again were not detected from soil in Japan but were detected in silkworm excrement. And the frequency of the detection of *A. flavus* group fungi and aflatoxigenic fungi increased from north to south. Cyclopiazonic acid was found to be produced by both aflatoxigenic and nonaflatoxigenic *A. flavus* group fungi. More wild strains of *A. flavus* group fungi are capable to produce CA than domesticated koji molds.

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