Original article

Laboratory studies on the outbreak of Gangrenous Ergotism associated with consumption of contaminated barley in Arsi, Ethiopia

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Abstract

Background: Ergotism is caused by the fungus *Claviceps purpurea*, which parasitizes cereal grains and is ingested by man through flour milled from contaminated cereals. An outbreak of ergotism in Ethiopia in 1978 resulted from exposure to ergot alkaloids from *C. puruprea* sclerotia.

Objectives: The objective of this study was to investigate consumption of cereal grains grown locally as the most likely cause of the outbreak of gangrenous ergotism so that control measures could be applied. **Methods**: During June to August, 2001, there were reports of a large number of cases of gangrene in Arsi Zone, Ethiopia. A multi-disciplinary team assessed the outbreak of the decease. Non-structured in-depth interviews were conducted with heads of households of the affected, and each of the patients was also interviewed. Grain samples were then collected from the interviewed households and analyzed for ergot alkaloids. Acute toxicity studies were also conducted by feeding male, non-pregnant and pregnant Swiss albino mice with the collected grain samples.

Results: Mycological cultures of grain samples yielded ergot alkaloids. All the grain samples contained ergot alkaloids, but with varying concentration. The highest concentration of ergotamine was observed in grain samples No. 4 (2.51 mg/100 g) and No. 6 (2.66 mg/100 g). Grain samples No. 2 and 7 had similar concentration of ergotamine, but more than four-fold higher than in grain sample No. 3. In contrast, the concentration of ergometrine in grain samples No. 4 (1.15mg/100 g) and No.6 (1.21mg/100 g) were twofold lower than ergotamine. The highest death (55%) of mice was observed in those test groups fed on grain samples No. 4 and No. 6. Cases of abortion were noted after 3 days of feeding in all pregnant mice with the exception of those allocated to grain sample No. 3.

Conclusion: We conclude on the basis of these results that the outbreak of gangrene in Arsi Zone, Ethiopia, is attributed to the ingestion of barley containing ergotized wild oats. [*Ethiop.J.Health Dev.* 2002;16(3):317-323]

Introduction

Ergot is the common name of the sclerotia of fungal species within the genus *Claviceps*, which produce ergot alkaloids. The sclerotium is a darkcolored, hard fungal mass that replaces the seed or kernel of a plant following infestation (1). Ergotism arises from ingestion of food prepared from grain contaminated with ergot fungus *Claviceps purpurea* or chronic use of drugs derived from this source. In man, ergot induces two types of epidemic ergotism; the gangrenous and convulsive forms. Gangrenous forms of ergotism are produced by ergotamine-ergocristine alkaloids of *C. purpurea* because of their vasoconstrictive activity. Major clinical manifestations include desquamation of the skin, feeble or absent peripheral pulses, edema, and dry gangrene of the limbs. The disease paralyzes the receptors at nerve endings, induces strong contractions of mediumsized arteries and small nutrient vessels and causes temporary or prolonged spasms which may ultimately lead to gangrene (2).

The last recorded outbreak of gangrenous ergotism in Ethiopia occurred in 1977-78 in Waro and GazoBelay sub-Woredas, Wedla-Delanta and Lasta Awrajas, Wollo administrative region with epidemic proportions, where 93 cases and 47 deaths were reported (3-5). The severity of infection in the mentioned area was observed on wild oats locally known as a '*ginche*' with ergot infestation ranging

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from 10-55%, but no ergot was observed on barley grains. The consumption of the affected '*ginche*' mixed with barley and other grains resulted in the development gangrenous ergotism (6).

The other type of ergotism, a convulsive form related to intoxication with clavine alkaloids from *C. fusiformis*, was seen during 1975 in India where 78 persons were affected. It was characterized by gastrointestinal symptoms followed by convulsion and other effects on the central nervous system (7,8).

Recently there was an outbreak of gangrene of unknown etiology in the Arsi Zone, Oromia Regional State. The outbreak occurred during February to June, 2001, following the new grain harvest in six Kebeles in Tijo-Digelu Woreda. Since the disease is uncommon in the area, Health Stations were not able to diagnose the disease and as the numbers of the affected increased Oromia Health Bureau requested for support in outbreak investigation. Hence, the objective of this study was to investigate consumption of cereal grains grown locally as the most likely cause of the outbreak of gangrenous ergotism in Tijo-Digelu Woreda, so that control measures could be applied.

Methods

Study area and population: A team consisting of the Oromia Health Bureau, the World Health Organization (WHO), Ethiopian Health and Nutrition Research Institute (EHNRI) and Medical Faculty, Addis Ababa University, was established to conduct the investigation. The survey was carried out during September, 2001, in Tijo-Digelu Woreda, about 70 km from Assella, the capital city of Arsi zone. Based on a review of past reports in the country and interviews with heads of households of affected families, a hypothesis that the outbreak was a food borne disease, due presumably to a chemical toxin or infectious agent was made.

Health workers from Tijo-Digelu Health Station have requested eighteen households that have previously visited the Health Station for treatment of the disease to report for review by the investigating team. Some of the households walked for more than four hours to the Station. Eleven of the eighteen households all accompanied by affected patients were available for the review.

Clinical examination of 13 patients was made by a physician from the investigating team. Two families had two cases each. However, their parents presented stories of two that died earlier.

The team conducted non-structured in-depth interviews with heads of households affected. The interview questions were designed to elucidate the following elements: knowledge about the disease, signs and symptoms of the disease and the number affected in each family, food preparation methods and food habits; any unusual quality of the harvest and weather changes observed, and types of crops grown in the area. Samples of grains consumed by seven households affected were collected whereas; the remaining households were inaccessible by car. Sampling of grains was from top, center and bottom of the granaries of the households affected. The samples were combined, homogenized and a composite sample of 1 kg was taken for analysis.

Sample preparation: Grain samples were examined for foreign matter following the methods described in the AOAC (9). A portion of the grain sample was finely ground without further cleaning in a standard Cyclotec1093 sample mill (Tecator, Hoganas, Sweden) to pass through 40-60 mesh and stored at 4^0C until analysis.

Chemical Analysis

Reagents

All the chemicals used were of analytical reagent grade, and the solutions were prepared in distilled water (DW). Ergometerine maleate (Lot No.94F01451) was purchased from Sigma (Sigma Chemical Co., St. Louis MI,USA) and ergotamine tartarate (Lot No.090055) was obtained from Pharbirth (Switzerland).

Reagent 1. Prepared by dissolving 0.5 g *p*dimethylaminobenzaldehyde (PDAB) in 50 ml mixture containing ethanol and concentrated sulfuric acid 60:40 ratio (v/v).

Reagent 2. (Modified van Urk's reagent). Prepared by dissolving 0.125 g PDAB in cold mixture of 65 ml concentrated sulfuric acid and 35 ml distilled water. To this 0.1 ml of 5% ferric chloride solution was added and left to stand overnight before use.

Ergotamine and ergometrine standard curves were drawn from stock solutions by dilution with distilled water and mixing with reagent 2.

Extraction mixture. Nine ml methanol + 1 ml concentrated ammonia + 90 ml chloroform.

Qualitative determination of ergot alkaloids:

Powdered grain samples were defatted first with petroleum ether (40-60 ^{0}C). The defatted residue was then basified with 10% ammonium hydroxide and extracted with diethyl ether which was concentrated under reduced pressure to small volume and mixed with 2 ml of 1% tartaric acid solution. Part of the solution was utilized for color test with reagents 1 and 2 using diethyl ethertartaric

acid mixture, standard ergotamine and ergometrine as controls. The remaining solution was used for additional confirmation of the ergot alkaloids using thin layer chromatography (TLC).

The plates were spotted alongside ergotamine and ergometrine standards and developed by the following solvent system: (i) Dimethlyamine: Ethanol: Chloroform (1:10:89 v/v/v), (ii) Chloroform: Methanol (95:5 v/v). After development, the plates were dried and sprayed with reagents 1 and 2 and developed at 110^0C for 15min. Ergot alkaloids appeared as blue or pink spots. The R_f (retarded factor) values were then compared with ergotamine or ergometrine standard spots.

Levels of fungal infestation were also determined in the food grains. Fungal growth on the grain samples was determined using direct plating of 100 grain kernels or flour on PDA (10). The alkaloids were extracted from the mycelial cells according to the method of Tejo Hernandez, *et al* (1992) and color test was performed to detect the presence of the alkaloids using reagents 1 and 2 (11). Ergotamine and ergometrine were used as standards, and distilled water as a blank.

Quantitative determination of ergot alkaloids: Ergot alkaloids were analyzed according to the method reported elsewhere, which can be briefly summarized as follows (12). Sixty grams of powdered grain samples were taken in triplicate and extracted with the extraction mixture (2x) followed by 50 ml chloroform and 4 ml distilled water. Each extract was then filtered, combined and dried *in vacuo*, and dissolved in 50 ml diethyl ether. This was further extracted with 15 ml of 0.2N H_2SO_4 (3x) followed by washing with 30 ml diethyl ether. The washed 0.2N H_2SO_4 extract was combined and diluted to 5 ml with distilled water. Ten ml of the extract was then taken and the color developed with 20 ml of reagent-2 or the van Urk's reagent. The absorbance was read after 30 minutes at 590 nm using a reagent blank. The standard curve was drawn by taking the absorbance value of different dilutions of standard ergotamine and ergometrine mixed with reagent-2.

Acute toxicity studies: An in-house breed of male and female Swiss Albino mice aged approximately 5 weeks with a narrow weight range (25-28 g) were used for the first experiment.

Three male and three female mice were allocated to each test diet. A further group of 14-18 days pregnant mice weighing 30-35 g were used for the second experiment. Five pregnant mice were allocated to each test diet. A further negative control group fed basal (normal) diet alone was included for each experiment. On receipt, the animals were weighed and assigned to treatment groups using a randomization procedure. The animals were housed in polypropylene cages with stainless-steel grid tops, suspended over paper for removal of excreta. Housing was in a room with ambient temperature and 12 hr light-12 hr dark cycle. The animals were kept in the study room for one day prior to the start of treatment to allow them to acclimatize to the environmental condition. Domestic main tap water was supplied *ad libitum* to each cage in glass bottles fitted with stainlesssteel lick spouts and rubber stoppers. Diets were fed *ad libitum* in glass feeders to the mice for a continuous period of at least 15 days. The animals were continuously observed until signs of convulsion, paralysis, restlessness, abortion, or death occurred (13).

Results

Results of physical examination of the grain samples indicated that sample No. 6 is highly contaminated with admixtures followed by samples No. 4, No. 1 and 5 (Table 1). Although ergotaffected barley or wheat kernels were not observed in the samples, the admixtures consisted of mostly wild oats (*sinar*) infected with ergot. However, heads of households interviewed during the investigation were not able to identity ergotized *sinar* in their grains, but they reported that the barley of 2000-2001 harvest has a peculiar odor previously not encountered and it contained unusually high wild oats (*sinar*).

Review of the ergotism cases in the survey area indicated that 18 patients aged 5 to 30 years were affected by the disease with three reported deaths (data not shown). Although no conclusive evidence of any sex difference in the susceptibility of children or adults to ergotism exist, children were the main victims of the disease in the present study similar to results of studies in Wollo (5). The present study also indicated that whole families were not affected by the gangrene, which may be attributed to the great variation in individual susceptibility of the affected households to particularly the gangrenous form of ergotism.

Table 1: **Physical examination of grain samples**[a]

Sample code	Type of grain	% weight of pure kernels	% weight of ergot, straw, etc.
1	Barely	92.35	7.65
2	Barely	94.17	5.83
3	Barely	97.75	2.25
4	Barely	64.31	35.69
5	Wheat	92.64	7.36
6	Barely	59.36	50.64
7	Barely	96.55	3.45

[a]The numbers given are the average of three determinations

Table 2 indicates results of qualitative analysis of ergot alkaloids in the grain samples. All the grain samples gave positive reaction with Reagent 1 and 2. Extracts of grain samples No.4 and 6 yielded intense color upon reaction with reagents 1 and 2. Similarly, extraction from mycelia cells cultured from the same grain samples gave similar intense colors. The results further indicate that all the grain samples contained ergot alkaloids in varying concentration as compared with the ergometrine and ergotamine standards.

Thin layer chromatography techniques (TLC) has also been applied to further confirm the presence of ergot alkaloids in the grain samples. As shown in Table 2, ergot alkaloids were identified by TLC in all the cereal grains. Grain samples and reference standards of ergometrine and ergotamine were matched by R_f values and color spots. Therefore, all results of the three techniques were in agreement on the presence of ergot alkaloids in all the cereal grains.

code	Sample extract	Mycelial extract	Sample extract	Mycelial extract	Solvent S ystem A B		Solvent s ystem	
1	Pink	Pink	Blue	Blue	0.43	0.62	0.13,	0.64
2	Pink	Pink	Blue	Blue	0.43	0.62	0.13.	0.64
3	Pink (f)	Pink (f)	Blue (f)	Blue (f)	0.43 (f)	0.62(f)	0.13 (f),	0.64(f)
4	Pink (i)	Pink (i)	Blue (i)	Blue (i)	0.43 (i)	0.62(i)	0.13 (i),	0.64(i)
5	Pink (f)	Pink (f)	Blue (f)	Blue (f)	0.43 (f)	0.62(f)	0.13 (f),	0.64(f)
6	Pink (i)	Pink (i)	Blue (i)	Blue (i)	0.43 (i)	0.62(i)	0.13 (i),	0.64(i)
7	Pink	Pink	Blue	Blue	0.43	0.62	0.13,	0.64
Standard Ergotamine	Pink	Pink	Blue	Blue		0.62 0.43		0.64 0.13
Standard Ergometrine	Pink	Pink	Blue	Blue				
Blank	Colorless	Colorless	Colorless	Colorless		NA		NA

Table 2: **qualitative determination of ergot alkaloids in grain samples**

Sample	Color test Reagent 1	R_f values on TLC Plates Reagent 2

Quantitatively, average concentrations of ergotamine and ergometrine in the cereal grains were determined by reactions with the modified van Urk's reagent and compared with standards (Table 3). The concentrations based on spectrophotometry for ergot alkaloids were in agreement with the TLC methods. As indicated in Table 3, the highest concentration of ergotamine was observed in grain samples No. 4 and 6. Grain samples No. 2 and 7 had similar concentration of ergotamine but more than four-fold higher than in sample No. 3. In contrast, the concentration of ergometrine in grain samples No. 4 and 6 were two-fold lower than ergotamine. These cereal grains have relatively high levels of ergot alkaloids as compared with the other samples.

Table 3: **Content of ergot alkaloids (ergometrine and ergotamine) in grain samples (mg 100 g of sample)** [a]

Sample code	Ergotamine	Ergometrine
1	0.57	0.26
2	0.91	0.41
3	0.21	0.09
4	2.5	1.20
5	0.29	0.19
6	2.7	1.20
7	0.93	0.43

[a]The numbers given are the average of three determinations

Table 4 indicates toxicity data of the cereal grains on mice. Convulsion, which began as muscle tremors and subsided within 2-4 minutes, was observed only in mice of both groups fed on samples No 4 and 6. In contrast, crippling and loss of movement was observed after 10 days of

Value in parenthesis are percentages

Discussion

The presence of ergotized wild oats locally named '*sinar*' in grain samples collected from Arsi Zone is consistent with a pervious study in Wollo where ergot was found to affect only the wild oat, which constitute a major portion of the harvested barley
(6). Ergot [*Claviceps purpurea* (**FT**) **Tu1**.] attacks rye, wheat, barley and many grasses. The disease is

easily identified when the conspicuous, blue-black ergots (sclerotia) are mature and give off a noticeable odor (14).

C. purpurea sclerotia produces ergoline (ergotamine and ergometrine) alkaloids (15). These alkaloids are characterized predominantly by gangrenous form of ergotism which act by contracting the medium sized arteries and small nutrient vessels, giving rise to gangrene of extremities, and muscle twitching (16).

The signs and symptoms observed in our study in mice after feeding the grain samples are remarkably similar to classical ergotism in animals. Applegrade (1986) reported that suckler cows all in late pregnancy, aborted within seven to 10 days following introduction to a rye grass pasture having infested with ergot, whereas Schneider *et al* (1996) observed an outbreak of gangrenous necrosis of the extremities in young cattle (17,18). Barley screenings containing ergotized annual ryegrass seed was identified in the later case as the toxic component and probable source of the ergot alkaloids in the ration.

Barley and wheat are the major crops cultivated in Tijo-Digelu Woreda. Wheat is usually a cash crop whereas, barley is the staple diet from which a number of food items are prepared. *Injera* prepared from barley is the staple food served at the main meals. Some households also take porridge, *kollo* or *kitta* (unleavened bread) prepared from barley or wheat. In the survey area barley or wheat is taken to local mills without further cleaning of the grains a practice which poses a high risk of ingesting the ergots by the population through flour milled from contaminated barley or wheat. Even the food preparation methods, for example the baking of *injera*, practiced by the housewives does not alter the alkaloids produced by ergot and ingestion of small amounts daily over a period of several weeks longer results in chronic poisoning (19).

During the survey households responded that there were weather changes during the preceding 2 to 3 years during which a combination of period of wet weather and hail late in the season were observed. These conditions were likely to favor the development of *Claviceps* species on grasses, in barley or wheat harvest, with the production of ergot alkaloids. Moist, cloudy weather during flowering favors fungal infection, because moisture favors the fungus and also because cereal or grass flowers remain open and succulent long under these conditions (20-21).

Our findings based on the chemical analysis of the cereal grains and toxicity data confirm that the outbreak of gangrene in Tijo-Digelu Woreda and the surrounding areas, Arsi Zone, is attributed to ergot alkaloid poisoning caused by consumption of barley or wheat contaminated with ergot-affected wild oats (*sinar*). The results are therefore, in agreement with previous studies in Ethiopia where the consumption of the mixed grain with the affected *ginche* (wild oats) has resulted in the development of gangrenous ergotism among the people living in Wadla-Delanta and Lasta Awrajas, Wollo (5).

The evidence provided in the present study that ergot alkaloids can cause an outbreak of disease in man further emphasizes the need for the government to formulate and implement strategies to control the development of *Claviceps* in agriculture.

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feeding in both test groups except for those fed on grain samples No-3 and 5. However, debilitation, early symptoms of restlessness, hypersensitivity of short duration, followed by depression were observed in both test groups of mice. The animals were overcome by paralysis, intermittent convulsive movement, then complete exhaustion
and loss of movement and gangrene was observed in the majority of mice following 10 days of feeding. Similarly, cases of abortion was noted after 3 days of feeding in all pregnant mice with the exception of those allocated to cereal grain No.3. The highest death (55%) of mice was observed in those test groups fed on grain samples No.4 and 6. The percentage of death of the test animals were however, similar for grain samples No. 1, 2 and 7. No abnormalities however, were seen in any of the mice from the control groups.

Table 4: **Acute toxicity studies**

Experiment	Toxicological effects observed on mice					
	Sample code	convulsion	Crippling & loss of movement	Debilitation, Restlessness, etc.	Abortion	Death
Experiment 1	1	Absent	Present	Present	NA	4 (36)
	2	Absent	Present	Present	NA	4 (36)
	3	Absent	Absent	Present	NA	2 (18)
	4	Present	Present	Present	NA	6 (55)
	5	Absent	Absent	Present	NA	2 (18)
	6	Present	Present	Present	NA	6 (55)
	7	Absent	Present	Present	NA	4 (36)
	Control	Absent	Absent	Absent	NA	Nil
Experiment 2	1	Absent	Present	Present	Present	4 (36)
	2	Absent	Present	Present	Present	4 (36)
	3	Absent	Absent	Present	Absent	2 (18)
	4	Present	Present	Present	Present	6 (55)
	5	Absent	Absent	Present	Present	2 (18)
	6	Present	Present	Present	Present	6 (55)
	7	Absent	Present	Present	Present	4 (36)
	Control	Absent	Absent	Absent	Absent	Nil

NA = Not Applicable
Experiment 1 = Three male and three female non pregnant mice were allocated for each test diet and control.
Experiment 2 = Five pregnant female mice were allocated for each test diet and control