FAO/UNEP/USSR

International Training Course

«TRAINING ACTIVITIES ON FOOD CONTAMINATION CONTROL AND MONITORING WITH SPECIAL REFERENCE TO MYCOTOXINS»

Z. A. KURBATSKAYA

BIOLOGICAL TESTS FOR INDICATION OF MYCOTOXINS

Centre of International Projects, GKNT

Moscow, 1984

BIOLOGICAL TESTS FOR INDICATION OF MYCOTOXINS

Kurbatskaya Z.A.

Many biological methods are used for determining the toxicity of foods and feedstuffs contaminated with toxin-producing fungi as well as for determining the toxicity of such fungi.

A review of some of these methods is presented.

Determination on Protozoa

Paramaecium candatum or Tetrahymena pyriformis are used as test-objects for primary screening of toxin-producing fungal cultures (both the known and non-identified ones), for assessing the toxicity of aqueous extracts of fungal mycelium, culture fluids and feed extracts. For this purpose pure homogeneous cultures of protozoa are used. The best nutrient media for the protozoa are hay infusion and lactic medium. Put motley grass hay into a flask, add some water and boil for 30 min. Keep the hay broth at a room temperature for 2-3 days for development of Bacillus subtilis and inoculate paramecia. In 7-10 days paramecia form a kind of thin film on the surface of the nutrient medium; 10-20-day cultures are used for testing.

The lactic medium may also be used for culturing protozoa. For this purpose add 2-3 drops of fresh skimmed milk to cooled boiled water. Lactic-acid bacteria proliferate in this medium and serve as feedstuff for protozoa. Pure cultures of paramecia should be maintained and inoculated into a new medium every 1.5-2 months.

Determination procedure. Place two drops (0.2 ml) of the test extract or culture fluid on a slide and inoculate a drop

(0.1 ml) of liquid with a definite number of paramecia into them. Mix all drops, note the starting time and put the slides into a moist chamber. Check the behavior of protozoa every 1-2 min for one hour, and then for 24 hours - at longer intervals.

The time from the onset of exposure to the test extract until the death of paramecia is considered to be the criterion of toxcity determination. Disorders in the behavior of paramecia and their death are certified after complete cessation of movement and the appearance of decay. The death of paramecia after exposure to fungal extracts or cultures takes place within max. 8 min after exposure to highly toxic samples, within max. 20 min after exposure to toxic samples and within max.2 hours after exposure to mildly toxic samples. Non-toxic fungal strains or extracts from the non-toxic test samples do not cause death or any morphological aberrations in paramecia even after 24 hours. Paramecia are highly susceptible to many mycotoxins.

Rabbit Skin Test

The rabbit skin test is one of the earliest and universally recognized methods for determination of toxicity of foods and feedstuffs contaminated with toxin-producing fungi. This method is based on a pronounced necrotic effect of toxic metabolites of many species of microscopic fungi on rabbit skin. This method is widely applied in the practice of toxicology.

Test procedure. Weight 50 g of finely ground sample (food, feedstuff or raw materials) into a Soclet extractor, extract the material with a solvent (ether, chloroform,acetone, ethyl acetate, alcohol, etc.) for two hours or in jars with ground

stoppers for 24 hours at the room temperature, shaking them from time to time; filter the solvent, collect it in evaporating dishes and evaporate under exhaust hood until the odour of the solvent disappears.

Skin test. Choose rabbits weighing not less than 1.5 - 2 kg with pigment-free skin and several hours before the test shave skin areas 4 x 4 cm on the sides, fore- and hind-extremities. Apply the obtained extract to the intact shaved skin and lightly rub it in with a spatula. Record the skin response in 24 hours and repeat the application. Put a collar (rubber, cardboard or veneer) on the rabbit neck to prevent licking the test substance off.

Registration of the reaction. The reaction should be recorded daily. The dermal inflammation appears on the skin the first or the second day after applying the extract, develops by the 4th - 5th, sometimes reaching its peak on the 7th day. The degree of toxicity of the test sample and a conclusion in respect to its rational use are assessed by the depth and severity of the skin injury after application of the extract. N.A. Spesivtseva suggested the following classification of the rabbit skin inflammation in response to the extract application:

the first degree - erythema and hypersensitivity of the skin, desquamation disappearing one or two days after application (the sample's toxicity is very low).

the second degree - erythema, tenderness, insignificant thickening of the skin, small solitary yellowish vesicles, later replaced by thin scabs of dried exudate, desquamation (the

sample is mildly toxic);

the third degree - considerable thickening and plication of the skin, tenderness, appearance of vesicles with exudate all over the treated areas, followed by surface dry necrosis, sometimes ulceration (the sample is toxic);

the fourth degree - erythema, strong edema protruding over the lower border of the focus, deep dry necrosis, appearance of protracted non-healing ulcers (the sample is highly toxic).

Determination on the Guppy (Lesbistes reticulatus)

The method is designed for determining toxicity in cereal feeds (grain and milling products) contaminated with microscopic fungi. For this purpose average samples of grain, milling products, flour, etc. are taken in conformity with the GOST (State Standard of the USSR) and are sent to the laboratory for examination.

The method allows to determine toxicity in cereal feeds within 24 hours.

Test procedure. Weigh 50 g of cereal feeds or milling products, comminute the material in the laboratory grinder and put into a flat-bottomed flask with a ground stopper, and 100 ml of acetone and extract by shaking on a shaker for 2 hours. Filter the extract through filter paper, collect it into a porcelain cup and evaporate to dryness (defatting with hexane). Dissolve the dry residue in 5 ml of acetone and transfer it into a vessel containing 500 ml of water taken from an aquarium (17-20°C), mix thoroughly and place five guppies with no regard to sex or

age into the solution. Observations are carried out for 24 hours; the time of guppy death is registered in 1, 2, 4, 8, 12, and 24 hours.

Assessment of the results. In relation to the degree of toxicity of the test sample the guppies die within periods stated in the Table below. 1% aqueous solution of acetone is used as control; guppies should remain alive in this solution for three days.

Assessment of the degree of toxicity of the test sample

Degree of toxicity	Dead guppies		Time of death in hours
	number	per cent	
Nontoxic	maximum one	up to 20	up to 24
I low	2-3	40-60	12-24
II medium	4	80	4-8
III high	5	100	2-4
IV very high	5	100	1

Determination of the tolerance dose on animals

Along with feeding of animals and direct intragastric introduction through a tube the test substrate extracts are injected intraperitoneally and intravenously for determining the degree of toxicity and assessing the quality of the samples. Mice, rats, guinea pigs and rabbits are used for this purpose. When working with animals it is imperative to observe the maximum permissible

quantity of fluid which can be administered via different routes. A single intramuscular injection to mice is 0.5-1 ml, a single or double injection to guinea pigs - 2-3 ml, a double injection to rabbits - 2-3 ml. By intubation into the stomach it is allowed to administer from 0.5 to 5 ml, and by intravenous injection - from 0.5 to 10 ml, depending on the animal species. Aqueous and alcoholic extracts (9°), containing mycotoxins or sterilized culture fluid are injected intraperitoneally in the amount of 0.5-1 ml to mice, 2-3 ml to guinea pigs, and 5 ml to rabbits.

For assessing the tolerance and toxicity of the preparation it is necessary to determine its maximum tolerance dose (MTD), the absolute lethal dose (LD_{100}), and the dose lethal for 50% of the animals (LD_{50}). The investigation should be started by determining these doses for albino mice. The text extract is administered in various quantities ranging from low doses, only poisoning the animals, up to high doses, resulting in their 100% death. In practical work one should proceed from some theoretical concepts, analogies or preliminary data for tentative plotting the order of different doses to be administered for LD_{50} determination: decimal parts, one or several mg/kg, mg/ml, etc. From 5 to 10 mice, each weighing 18-20 g, should be taken for testing each dose injected intraperitoneally. The animals should be marked and continuously observed; it is necessary to record their behavior, weight and, if required, to check their blood, to note the time and number of animals that died from each particular dose. After administering toxic samples animals may die within a period from several hours up to 2 days. Slightly to-

xic substances may cause the death of animals after 6-12 days.

Calculation of the LD_{50} is carried out according to Kerber, his method being sufficiently simple and reliable.

The following equation is used for the calculation:

$$aM = Dm - \frac{\sum(z \cdot d)}{m}$$

where aM - LD_{50},

Dm - a dose which caused the death of all experimental animals;

Z - half of the sum of the number of animals which perished after two successive doses;

d - difference between the values of two successive doses;

m - number of animals per does;

$\sum$ - sum.

Example of LD_{50} calculation

Dose mg/ml	Number of animals per dose	Number of perished animals	Z	d	Z·d
0.9	5	5	-	-	-
0.8	5	4	4.5	0.1	0.45
0.7	5	2	3.0	0.1	0.30
0.6	5	4	3.0	0.1	0.30
0.5	5	3	3.5	0.1	0.35
0.4	5	1	2.0	0.1	0.20
0.3	5	0	0.5	0.1	0.05
					$\sum$ -1.65

$$aM = 0.9 - \frac{1.65}{5} = 0.57 \text{ mg}$$

Then the dose is recalculated per animal body weight (mg/kg).

G.N.Pershin suggests the following formula to calculate the average lethal dose of the test substance:

$$X_{av} = \frac{(a+b)\cdot(m-p)}{200},$$

where a+ b - sum of successive doses;

m-p - difference between the lethality percentage resulting from the two successive doses;

x_{av} - average lethal dose.

Example of calculation of an average lethal dose (X_{av})

Dose mg/kg	Number of animals	Number of perished animals	Mortality per cent	Average lethal dose mg/kg
20	10	0	0	
30	10	0	0	
40	10	4	40	43
50	10	8	80	
60	10	10	100	
70	10	10	100	

Calculation: 1. (30+40) x (40-0) = 2800

2. (40+50) x (80-40) =3600

3. (50+60) x (100-80)=2200

Sum = 2800 + 3600 + 2200 + 8600

$$X_{av} = \frac{8600}{200} + 43 \text{ mg/kg}$$

All experimental animals (which died or survived)are subjected to post-mortem examination and the pathological findings are recorded. The pathomorphological deviations in the organs and tissues are studied if necessary.

LITERATURE

1. Methodological instructions on sanitary and mycological examination of feeds. Moscow, "Kolos" Publishing House, 1970, pp.27.

2. Spesivtseva N.A. Mycoses and mycotoxicoses. Moscow, "Kolos" Publishing House,,1964, pp.520.

3. Methods of investigations in veterinary mycology. Edited by Spesivtseva N.A. Moscow, "Kolos" Publishing House, pp.311.

4. Methods of experimental mycology Edited by Bilai V.I., Kiev, "Naukova Dumka", 1982, pp.550.

Зак. 1226 ПИК ВИНИТИ