

Monitoring antibiotic residues and aflatoxin M1

- ✓ Antibiotics are used in dairy cattle management for preventing mastitis, brucellosis etc.
- ✓ Commonly used antibiotics in dairy animals: β -lactam, tetracycline, amino glycoside, sulfonamide and macrolides.
- ✓ **β -lactam**: Major group of antibiotics used for treatment of dairy cattle.
- ✓ Antibiotic residues in milk are allergic, carcinogenic and cause development of antibiotic resistant pathogenic strains .
- ✓ Presence of antibiotics residues in milk: Adverse affects during processing of dairy foods in terms of starter failure, poor ripening of cheese.
- ✓ Maximum residual limit (MRL) has been recommended for antimicrobial agents for their compliance in milk

Family	Example
Carbohydrate(amino glycoside) antibiotics	Streptomycin and neomycin
Macrocyclic lactone (Macrolides)	Erythromycin, oligomycin, amphotomycin
Amino acid peptide and β -Lactam antibiotics	Penicillin, polymixin, bacitracin, cephalosporin,
Acyclic antibiotics	Streptovitacin
Aromatic antibiotics (polyenes, antifungal)	Chloramphenicol, griseofulvin
Aliphatic antibiotics	Variotin
Quinine and similar antibiotics	Tetracycline

List of antibiotics used in dairy husbandry

Anti-microbial agents	MRL in milk
Benzyl penicillin	0.004 mg/ kg
Ceftiofur sodium	0.1 mg/ kg
Dihydro- streptomycin and streptomycin	0.2 mg/ kg
Gentamicin	0.1 mg/ kg
Neomycin	0.5 mg/ kg
Oxytetracyclin	0.1 mg/ kg
Pectinomycin	0.2 mg/ kg
Spiramycin	0.1 mg/ kg
Sulfadimidine	0.025 mg/ kg

Maximum Residual Limit (MRL) recommended for milk

Detection Methods for Antibiotics and Drug Residues in Milk

**Microbial
growth inhibitor
assay**

**Microbial receptor
assay**

**Enzyme-
colorimetric assay**

**Receptor binding
assay**

**Spectrophotometer
assay**

**Chromatographic
methods**

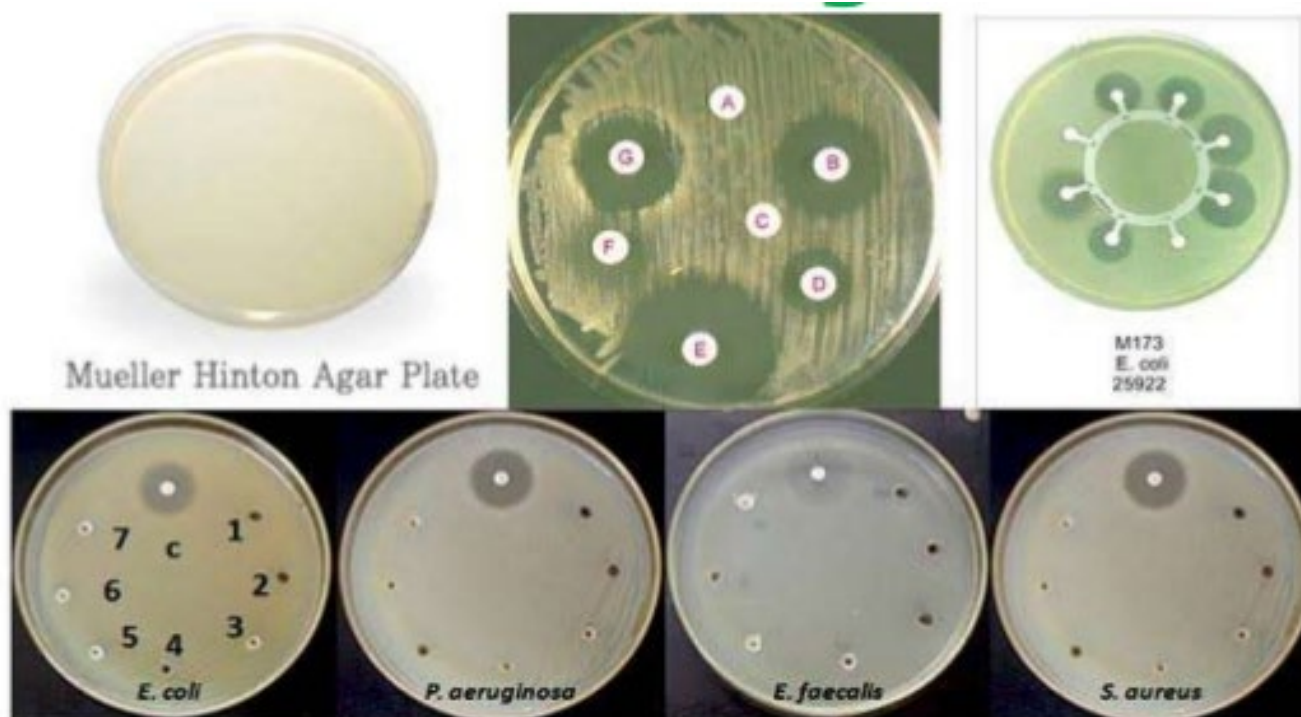
Immunoassay

Reference method

1. EU reference method for determination of antibiotic residues in raw milk and in heat-treated milk is the International Dairy Federation (IDF, 1991) microbial inhibition test.
2. IDF microbial inhibitor test uses *B. stearothermophilus* var. *calidolactis*, ATCC 10149 as the test organism due to its relatively high sensitivity to inhibitory substances.
3. Quite complex and lengthy to carry out as it involves the continual growth of large quantities of *B. stearothermophilus* spores.
4. As test involves a colour change, which is dependent on growth of *B. stearothermophilus*, if organism fails to grow then false negative result may occur.
5. **Microbial inhibitor test kit assays** based on IDF method are routine methods used for determination of antibiotic residues in milk.

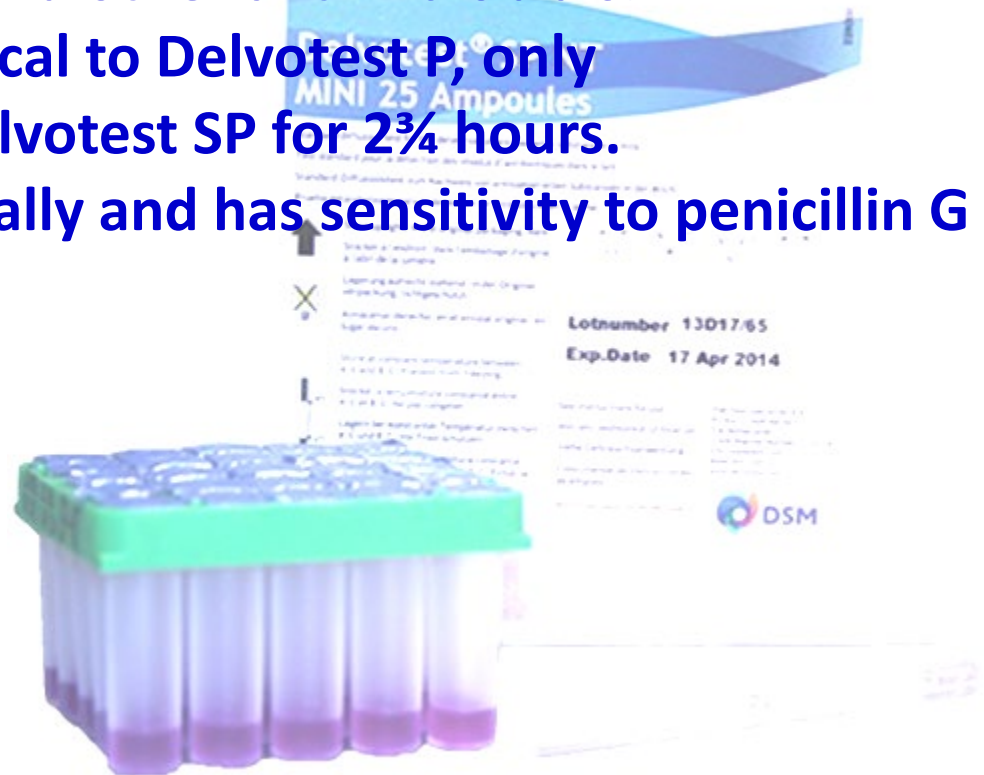
Microbial inhibitor test

1. Involve incubating a susceptible organism in presence of milk sample.
2. In absence of an antibiotic, organism grows and can be detected visually either by opacity of agar growth medium or by a color change resulting from acid production.
3. In presence of an antibiotic, or any other inhibitor, the organism fails to grow and a zone of inhibition or lack of a color change is observed.
4. Sensitive to β -lactam antibiotics.



a. Delvotest SP:

- Delvotest developed by Gist-brocades BV, The Netherlands is the best known microbial inhibitor test.
- Delvotest P has sensitivity to penicillin G of 0.005 IU/ml.
- Delvotest SP, is capable of detecting sulphonamides, but also has increased sensitivity to tylosin, erythromycin, neomycin, gentamicin, trimethoprim and other antimicrobials.
- Delvotest SP appears identical to Delvotest P, only difference is to incubate Delvotest SP for 2¾ hours.
- Delvotest SP is sold universally and has sensitivity to penicillin G of 0.003-0.004 IU/ml.



- **Test procedure**

Growth of *B. stearothermophilus* spores at 64°C initiates an acidification process which causes turning of a pH indicator from purple to yellow.

- **Presence of antibacterial substances will cause delay or inhibition of the spores, depending on concentration of residues.**
- **In presence of residues, spores will not multiply and pH indicator will remain purple.**



Steps are involved in procedure:

1. Add 1 nutrient tablet to each of the agar wells in the strip.
2. Inoculate 100 μ l of milk into agar well plus nutrient tablet.
3. Seal the wells for incubation.
4. Incubate strip of wells in a water bath at $64^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 2.5 h (negative control has been changed to yellow)
5. Examine the strip for colour change from purple to yellow. **Yellow reading indicates that no inhibitory substances are present; purple reading indicates that antibiotic residues are present and yellow/purple reading indicates a doubtful result.**

b. Copan test:

- Determine antibiotic residues in milk.
- The nutrient tablet is already added to agar medium.

c. Charm farm test

- Microbial inhibition test which uses a one step single service vial.
- Broad screening assay beta-lactams, sulphonamides, tetracyclines, aminoglycosides and macrolides in raw, commingled, bovine milk.
- Results are stable upto 8 h after assay completion and can be read by visual colour comparison.
- Charm Auto-Farm Equipment is required to run this test.
- Test can be completed in approx. 3.5 h.



d. Charm AIM-96:

- Designed for high volume, broad spectrum screening of raw, pasteurised, and homogenised or skim milk.
- Results can be read by visual colour comparison or optionally with a microplate reader.
- Charm AIM-96 detects beta-lactams, sulphonamides, tetracyclines, aminoglycosides and macrolides.
- 96 tests can be completed simultaneously in approx. 4 hours.

e. MDR test

- Sporulation & activation of dormant spores of *B. stearothermophilus* in newly developed medium & their germination/ outgrowth in presence of selective germinant mixture has been developed and is available commercially as microbial drug residue (MDR) test in India.
- Used effectively for semi-quantitative detection of antibiotic residues in different types of milk within 2.30-3.0 h at MRL/ or above levels as recommended by codex.

Immune-receptor test

- 1. Variation of well-established enzyme-linked immunosorbent assay (ELISA).**
- 2. Specific target antibiotic group is captured by immobilized antibodies, or by broader spectrum receptor such as bacterial cell.**
- 3. Antibody-antibiotic complex is then usually linked to an enzyme that catalyses a color or fluorescence reaction and comparison of intensity of 'test' reaction with that of 'control' determines whether sample is positive or negative.**
- 4. Low intensity means 'positive' whilst high intensity is 'negative'.**
- 5. Immune receptor tests can be made quantitative but are generally used to provide a 'pass/fail' result. Generally more expensive than microbial inhibitor tests but only detect substances that react immunologically with immobilized receptor and they provide a result in <10 min.**

- Commercially available immune receptor tests employ several variations of capture mechanism and color reaction but most possess common features of an immunological reaction coupled with change in color (or fluorescence).
- Two exceptions: **The Penzym test** (UCB Byproducts, Belgium) employs inhibition of an enzyme reaction (DD-carboxypeptidases activity), instead of an immune reaction, to detect presence of β -lactam and visualizes by a color change. Test produces pink color when a sample contains no antibiotics while, yellow color is interpreted as positive.
- Conversely, **Charm II assay** (Charm Sciences Inc., USA) employs an immune reaction to bind antibiotic to microbial receptor but detects this complex using low-level ^3H or ^{14}C radio-label, instead of an enzyme reaction. Suitable for large laboratories only, requiring centrifuge and sample mixers to prepare samples as well as a scintillation counter to detect the radio-label.

Novel iodometric test

1. Principle of spore germination and induction of β -lactamase enzyme in presence of inducer i.e. specific β -lactam group in milk.
2. When specific group i.e. β -lactam is absent, induction of β -lactamase enzyme during germination and production of penicilloic acid as a result of enzymatic action will be minimal resulting in no color change of starch & iodine mixture.
3. In presence of inducer there will be reduction of starch iodine mixture as a result of significant induction of marker enzyme and production penicilloic acid.
4. Change in color of starch iodine mixture from blue to colourless will indicate presence of β -lactam group in milk when incubated at 35 C for 15-20 min.

1 Control Test
- +
Add 1.5 mL of milk into clean test tubes

2 Control Test
- +
Transfer 450 ± 50 µl of spore suspension

3 Control Test
- +
Add 20 ± 5.0 µl of starch & 45 + 5.0 µl of Iodine solution

4 Control Test
- +
Incubate at 35 ± 2°C for 15-20 mins.

5 Control Test
- +
Observe for difference in intensity of colour

6 Control Test
- +
Reduction in intensity of colour (presence of β -lactam)

Detection of β -Lactam group in milk based on induction principle

Novel features:

**Result within 15-20
min**

Cost effective

**Semi-quantitative
detection at Codex
MRL**

**No cross reactivity
with non β -Lactam
groups**

**Validated with
AOAC approved
charm 6602 assay**

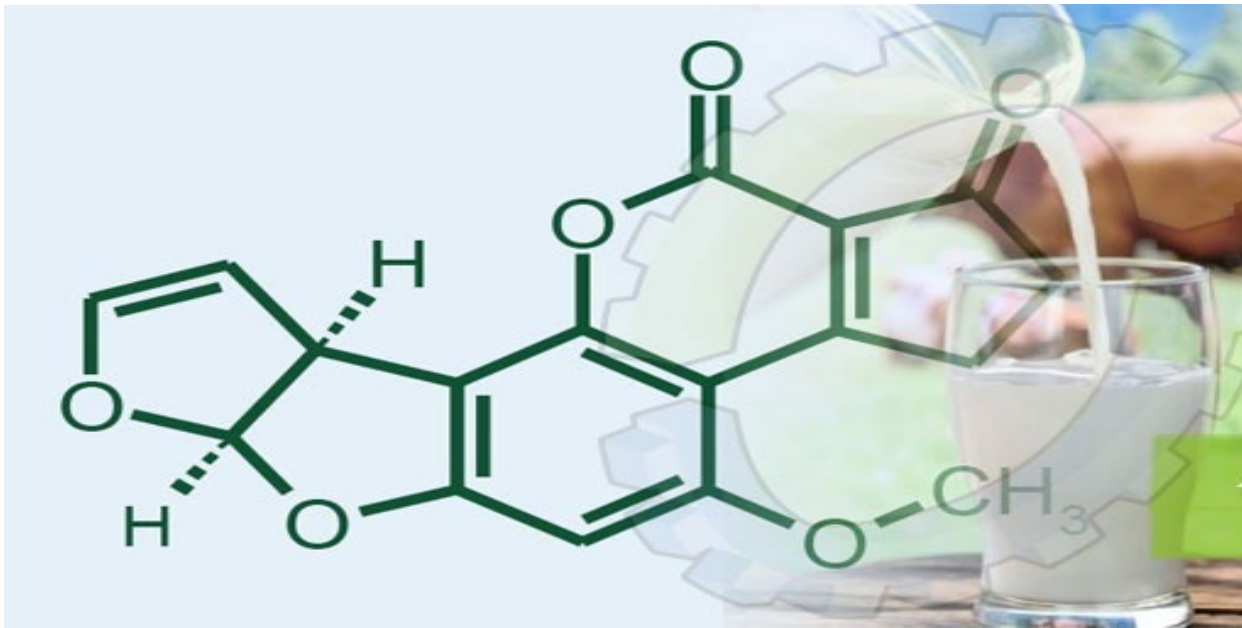
**Stability of test kit
up to 7-8 months
under refrigeration**

**Application with
raw, pasteurized
and dried milks.**

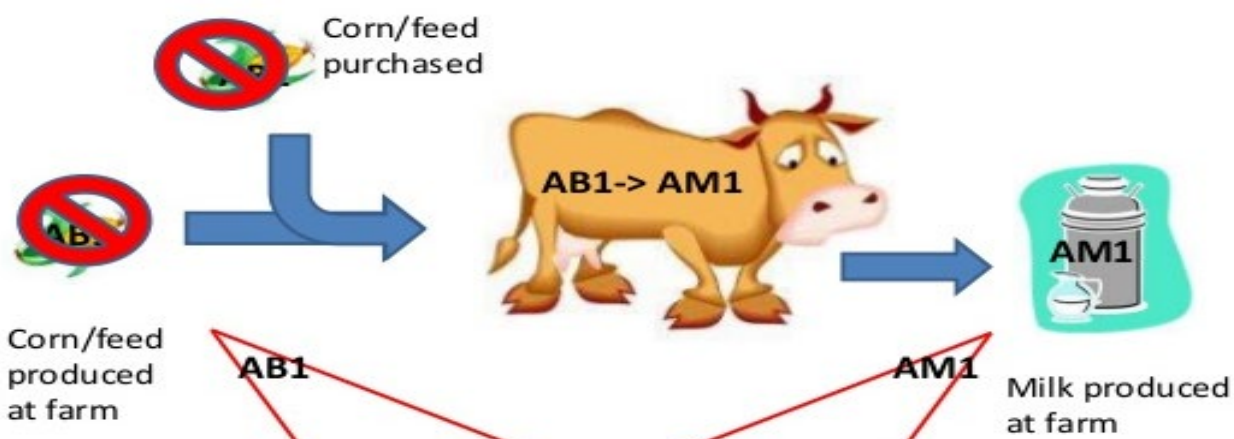
Test Methods for Aflatoxin M1 in Milk

1. Aflatoxins are toxic metabolites produced by certain fungi in/on foods and feeds.
2. Associated with aflatoxicosis, in livestock, domestic animals and humans.
3. Potent carcinogenic effect in susceptible laboratory animals and their acute toxicological effects in humans.
4. Chemically, defined as di-furano-cyclo-pentano-cumarines or di-furano-pentano-lido-cumarines, i.e. aflatoxins contain a dihydrofuran or a tetrahydrofuran ring, to which a substituted coumarin system is condensed.
5. Out of about 20 known aflatoxins, *Aspergillus flavus* and *A. parasiticus* produce exclusively aflatoxin B1, B2, G1 and G2, and all the other aflatoxins are derivatives of these four aflatoxins.
6. Aflatoxin M1 was the first metabolite of Aflatoxin B1, which could unequivocally be detected in the milk.

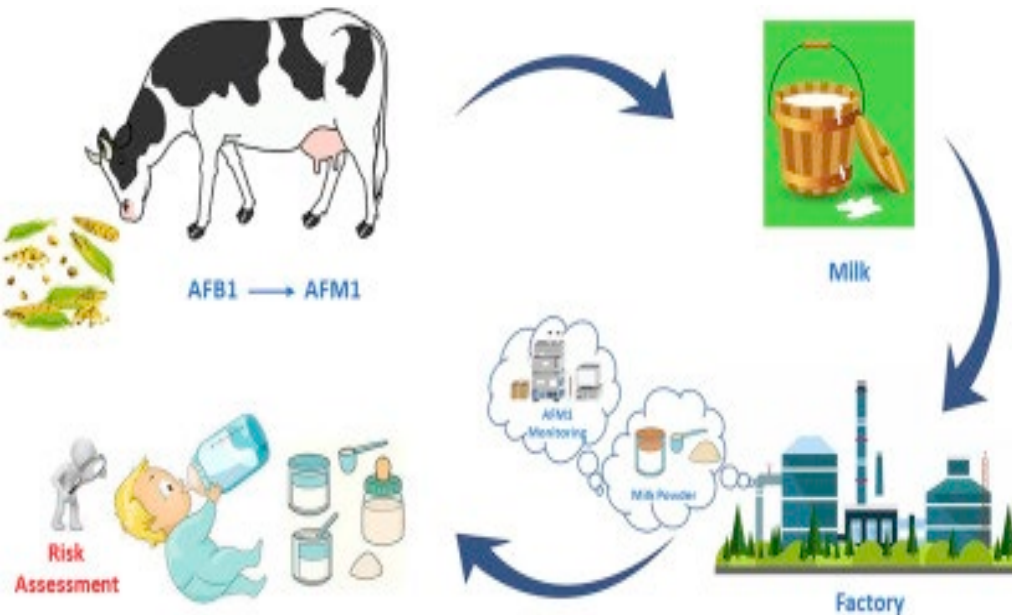
7. First derivative was called Aflatoxin M1 (milk).
8. When cows are fed contaminated feed, aflatoxin B1 is converted by hydroxylation to aflatoxin M1, which is subsequently secreted in the milk of lactating cows.
9. Aflatoxin M1 is quite stable towards pasteurization and if present in raw milk, it may persist into final products for human consumption.
10. In the codex, limit for the presence of M1 in milk is set at **0.5 $\mu\text{g}/\text{L}$ or 0.5 parts per billion (ppb).**



Aflatoxin flow



Human exposure



Aflatoxin M1 contamination results from conversion of aflatoxin B1 that is metabolized by enzymes found in liver. After aflatoxin M1 is formed, it is excreted in urine and milk of cow. Action level for aflatoxin B1 is 20 parts per billion (ppb) for feed fed to lactating dairy cows. Aflatoxin B1 in feed is mycotoxins produced by *Aspergillus* that grow on corn, cotton seed and peanuts. Feed does not contain aflatoxin M1 as it is found only in milk.

Rapid screening methods

Detection Method	Mechanism	Reaction Type	Time (Minutes)	Sensitivity (ppb)
Charm-II (radioimmunoassay)	Binding of radio labeled Aflatoxin with microbial receptor sites	Color Change	16	0.25 – 0.5
ELISA (RIDA screen)	Ag-Ab reaction	Color Change	70	50
HPLC with fluorescence detector	Immunoaffinity columns containing antibodies against AFMI	Fluorescence	60	6
Electrochemical immunosensor	Ag-Ab with electrochemical transducer	Color Change	75	30-160
Flow injection immunoassay	Ag-Ab with amperometric transducer	Color Change	> 60	20-500
Chemiluminescent enzyme immunoassay	Ag-Ab	Luminescence	80	1
Long range surface Plasmon enhanced fluorescence spectroscopy	LRSPFS with inhibition immunoassay	Fluorescence	53	6

Commercially available methods for aflatoxin M1

Spore based assay for aflatoxin M1:

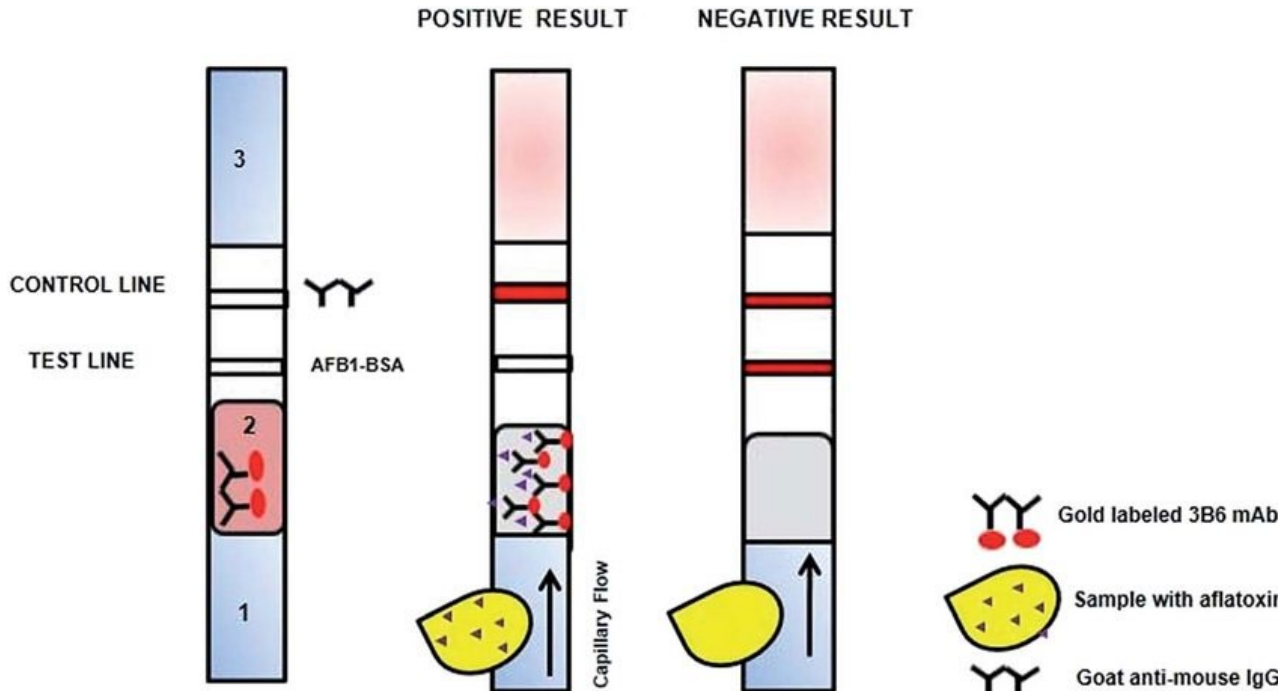
1. Bacterial spores have unique ability to sense environmental changes in response to specific “germinant” and transform rapidly into growing vegetative cells.
2. This characteristic can be effectively used as biosensor for tracking microbial and non-microbial contaminants.
3. Test based on specific spore germination and its inhibition in presence of specific analyte, i.e., aflatoxin M1 has been developed.
4. In case where analyte is absent in milk, specific indicator enzyme (s) are produced by active bio-sensing molecules which will act specifically on chromogenic/or fluorogenic substrate resulting in colored reaction/or fluorescence as end product which is measured semi-quantitatively by either visually/or using optical system at specific excitation/emission spectra.

Enzyme linked immune sorbent assay (ELISA):

- 1. Biochemical technique for detection of an antigen in a sample.**
- 2. Sandwich ELISA utilizes two antigen specific antibodies, a capture antibody bound to solid phase and an enzyme linked detection antibody.**
- 3. Direct enzyme conjugation of detection antibody ensures an easy-to-use and sensitive assay with minimal background signal.**
- 4. A competitive assay in which there is competitive binding of an antigen-specific, biotin-linked antibody to sample antigen or to antigen bound to microtiter well.**
- 5. Bound antibody is detected with enzyme-linked streptavidin.**

Lateral flow assay:

- 1. Charm Safe Level Aflatoxin M1 Quantitative (SLAFMQ) test is a colloidal gold lateral flow immunoassay.
- 2. Aflatoxin M1 in a milk sample competes with the antibody gold beads for binding to 2 test lines. Remaining unbound binder forms on control line.
- 3. Test and control lines are compared with reflectance reader, and ppt concentration is determined with an algorithm.
- 4. Negative interpretation with reading of [less than or equal to] 400 ppt and positive interpretation with reading >400 ppt was designed to detect 500 ppt.



Charm II test for aflatoxin (competitive assay):

- 1. Add 300 μ L solution AF to test tube**
- 2. Fill tube 3/4 full with milk sample or standard**
- 3. Centrifuge the sample at 3400 rpm for 5 mins**
- 4. Cool to 4 C**
- 5. Add white tablet to empty test tube**
- 6. Add 300 \pm 100 μ L water**
- 7. Mix 10 secs to break up tablet (take additional time if required to be sure tablet is broken up)**
- 8. Add 5.0 \pm 0.25 mL centrifuged sample or standard from below fat layer (new tips for each sample, milk temp. 4 C)**
- 9. Immediately add purple tablet**
- 10. Immediately mix by swirling milk up and down 15 times for 15 secs**

- 11. Incubate at 37 C for 3 minutes**
- 12. Centrifuge at 3400 rpm for 5 mins**
- 13. Immediately remove from centrifuge and pour off milk**
- 14. Remove fat ring wipe dry with swabs. Do not disturb pellet**
- 15. Add 300 ± 100 μL water**
- 16. Mix thoroughly to break up pellet**
- 17. To one tube at a time, add 3.0 ± 0.5 mL scintillation fluid.**
- 18. Cap, invert (or shake) until mixture has uniform cloudy appearance**
- 19. Immediately count in analyzer for 60 secs.**
- 20. Read CPM (count per minute) on (^3H) channel**