DETECTION OF INHIBITORY SUBSTANCES IN MILK

Bacillus stearothermophilus Disc Assay (BsDA), Charm Tablet Method (Raw Commingled Cow Milk, Raw Commingled Goat Milk, and NCIMS Accepted Pasteurized Cow Milk Products) IMS# 9-B2

[Unless otherwise stated all tolerances are ±5%]

GENERAL REQUIREMENTS

1. Laboratory Requirements (see Cultural Procedure (CP), items 33 & 34), except _____

a. For Appendix N testing, see Appendix N General Requirements (App. N GR), items 14 & 15 _____

SAMPLES

2. See CP, item 33, except _____

a. For Appendix N testing, see App. N GR items 9 _____

APPARATUS & MATERIALS

3. See CP items 1-23, except _____

a. For Appendix N testing, see App. N GR items 1-8 _____

4. Equipment _____

a. Incubator thermostatically controlled at 64±2°C _____

b. Heating block, water bath or other acceptable method to heat to at least 82±2°C, for confirmation _____

c. Pipettor - 90 µL and 500 µL (optionally 50 µL) and disposable tips (see App. N GR item 7 or CP item 6) _____

d. Forceps, Fine Points, Stainless Steel _____

e. Vernier, Dial or Digital Calipers, metal (readable to 0.1 mm) _____

f. Stirring hot plate/stirring bar (optional) _____

g. 100 mL Class A graduate cylinder _____

h. 250 mL Erlenmeyer flasks _____

i. 13 x 100 mm test tubes for beta-lactam confirmation _____

j. Timer _____

MATERIALS

5. See CP, items 24-32 _____

a. Filter Paper Discs, Blank, Unimpregnated, Non-sterile _____

Brand: _____ Lot #: _____ _____

1. High absorbability, diameter 12.7±0.1 mm _____

b. Charm PM Indicator Agar _____

1. **Do Not Autoclave** - (see plate preparation, item 19 below) _____

c. Charm Spore Tablets _____

1. Bacillus stearothermophilus (Geobacillus stearothermophilus) tablets containing 100,000,000 (±10 million) spores per tablet _____

Lot #: _____ Exp. Date: _____ _____

d. Plastic Petri dish (15 x 100 mm, bottom plate inner diameter 86.1 - 87.0mm) _____

REAGENTS

6. Reagents _____

a. Charm 5.0 ppb Penicillin G Standard Positive Control _____

Lot #: _____ Exp. Date: _____ _____

1. Store according to label directions _____

2. Rehydrate according to label instructions _____

3. Test for suitability each time prepared, add to one (1) disc, must produce zone 16-20 mm; maintain records _____

Avg. Zone Size: _____ _____

4. Use rehydrated standard within 48 hours if refrigerated _____

Lab Prep. Date: _____ _____

5. Or, aliquot within 24 hours and freeze at -15°C or colder in non-frost-free freezer or in an insulated foam container in a frost-free freezer, use within 2 months (Once thawed, maintain according to manufacturer's instructions) _____

   Lab Prep. Date: _____ Lab Exp. Date: _____ _____

b. Negative Control _____

1. Charm Zero Control Standard _____

   Lot #: _____ Exp. Date: _____ _____

   a. Reconstitute according to label instructions _____

   b. Use rehydrated negative control within 72 hours if refrigerated _____

      Lab Prep. Date: _____ Lab Exp. Date: _____ _____

   c. Or, aliquot within 24 hours and freeze at -15°C or colder in non-frost-free freezer or in an insulated foam container in a frost-free freezer, use within 2 months (Once thawed, maintain according to manufacturer's instructions) _____

      Lab Prep. Date: _____ Lab Exp. Date: _____ _____

2. Inhibitor Free Raw Milk _____

   Sample ID: _____ Date Tested: _____ _____

   a. Use within 72 hours if refrigerated _____

      Lab Prep. Date: _____ Lab Exp. Date: _____ _____

   b. Or, aliquot within 24 hours and freeze at -15°C or colder in non-frost-free freezer or in an insulated foam container in a frost-free freezer, use within 2 months. Once thawed, use within 24 hours _____

      Lab Prep. Date: _____ Lab Exp. Date: _____ _____

3. Test for suitability, add to one (1) disc, produces no zone; maintain records _____

   Zone Size: _____ _____

c. Charm Beta-lactamase tablet or liquid concentrate (not required if beta-lactamase is not used for confirmation) _____

1. Stored at -15°C or below _____

2. Do not use beyond expiration date _____

Lot #: _____ Exp. Date: _____ _____

3. Reconstitute freeze dried concentrate as per manufacturer instructions _____

a. Liquid concentrate stored at -15°C or below in a non-frost-free freezer or in an insulated foam container in a frost-free freezer; use within 2 weeks _____

4. Test each lot for suitability, add beta-lactamase to 5.0 ppb positive control (item 6.a) and add to one (1) disc, beta-lactamase neutralizes zone produced by positive control; maintain records _____

Zone Size: _____ _____

ASSAY PLATE

7. Preparation of Plate _____

a. Prepare agar according to label, 3.2 g/95 mL H_2O, bring agar to a boil _____

b. Promptly cool to 64±2°C (Temperature Control [TC] used) _____

1. Optionally, temperature may be determined by inserting a dedicated thermometer (not used for any other purpose) directly into test agar _____

c. Add 1 spore (white) tablet to 5 mL deionized water in 13 x 100 mm test tube _____

d. Shake test tube 25 times through 1 foot arc in 7 sec, or vortex for 10 sec; let settle 1 min _____

e. Repeat item d above _____

f. Decant spore mixture into agar tempered to 64±2°C leaving residue on bottom of tube (avoid pouring mixture down side of flask) _____

g. Mix agar well for 1.5 min avoiding incorporation of air bubbles, optionally use stirring bar on magnetic stir plate _____

h. Constantly mix remaining agar during preparation of plates _____

i. Pipet 6 mL inoculated agar into Petri dish (item 5.d) _____

1. Or, appropriate amount of agar into other size [$(Dcm)^2$ $6/8.65^2$ = V]; Dcm = inner diameter of plate in centimeters; V = volume (mL) of agar to add in dishes; maintain records _____

j. Plates have flat bottoms and do not buckle after addition of agar; plates observed before and after preparation for suitability _____

k. Swirl plate gently on level surface to evenly distribute agar _____

l. Allow agar to solidify on a level surface for 15 min with lid ajar _____

m. Use within 5 days, if stored at 0-4.5°C in airtight container _____

Lab Prep. Date: _____ Lab Exp. Date: _____ _____

TECHNIQUE

8. Performance Check (see App N. GR item 10.a) _____

a. Positive and negative controls give appropriate zones prior to any sample analysis (refers to new lot numbers) _____

b. Take corrective actions for out of range zones _____

c. Maintain records _____

9. Laboratory Procedure, Screening _____

a. Label bottom of plates prior to adding discs, use template as a guide to assure discs will be placed at least 10 mm from the Petri dish wall and other discs _____

b. Each test plate may contain a maximum of 5 test sample discs plus a positive control and negative control disc (7 discs total as per template, for larger plates more discs may be placed, maintain comparable spacing) _____

c. Sample agitation _____

1. Mix raw milk sample(s)/control(s) (approx. ¾ full), subsample(s) of retail milk containers or control(s) by shaking 25 times in 7 sec with a 1 ft movement or vortex for 10 sec at maximum setting; use within 3 min (samples/controls must be in appropriate container to allow the use of vortexing) _____

2. Mix retail samples by inverting containers top to bottom then bottom to top (a complete half circle or 180 degrees) without pausing, 25 times; use within 3 in _____

d. Add 90 µL of mixed sample/control to each disc _____

1. Using pipettor (item 4.c) with new tip for each sample/control, draw up 90 µL avoiding foam and bubbles _____

2. Remove tip from liquid _____

3. Using clean, dry forceps, remove a disc from container and place the disc (using a template as a guide) on the agar surface of the inhibitor plate _____

4. Press the disc **gently** with the forceps to insure good contact and then fill disc immediately _____

5. With pipettor in vertical position and tip about 5 mm above the center of the disc, depress the plunger to the first stop in such a way to get a rapid drop-wise release of the sample _____

   a. If pipettor has two (2) stops, depress plunger to second stop _____

6. Sample not applied too slowly or quickly (streamed) _____

7. Allow a second or two for the milk to absorb into the disc _____

8. **Gently** touch off the tip on an area of the disc away from where the sample was deposited _____

9. Repeat the above until all samples have been done _____

e. Place a positive control disc containing 5.0 ppb Penicillin G and a negative control disc on each test plate using above procedure _____

1. Vary the location of positive control discs in a series of test plates; i.e. center or outside of the plate _____

f. Invert plate(s) and incubate at 64±2°C until well defined zones of inhibition are obtained around the 5.0 ppb positive control(s), the remainder of the plate(s) should be yellow with an incubation time of approximately 2.5 to 3 hours _____

g. Remove plates from incubator and allow to cool on a level surface for 2 minutes (do not remove lid before plates are cooled) _____

h. Examine positive control zone. A valid test requires a positive control zone of 16-20 mm. If zone size is < 16 or > 20 mm the test **must** be repeated _____

i. Examine plate for zones of inhibition surrounding the test discs, zones of >12.7 mm indicates presence of inhibitory substances _____

j. Measure zones of inhibition by using calipers _____

1. Use the inside diameter points (smaller points) _____

2. Anchor one point in the bottom of the plate at the edge of the zone and expand calipers until the other point rests on the other edge _____

3. Read calipers and report zone size to the nearest 0.1 mm _____

k. Zones of ≤12.7 mm are read as no zone _____

l. Zones >12.7 mm must be promptly confirmed to report as positive for inhibitor or beta-lactam residue _____

10. Confirmation of PMO Section 6 Samples or Verification of Appendix N Initial Positive Tanker Samples (see App. N GR item 11); Confirmation of Presumptive Positive Tanker Samples (see App. N GR item 12); and if applicable, Traceback of Producer(s) on a Confirmed Positive Tanker (see App. N GR item 13). PROMPTLY retest the SAME sample in DUPLICATE along with a positive and negative control as described below (10.a.1-8) _____

a. Inhibitor confirmation/verification and optional beta-lactamase confirmation _____

1. Confirmation (without beta-lactamase) _____

a. Heat a 0.5 mL (500 µL) portion of each suspect sample to 82±2°C for 2 minutes (TC required) _____

b. Cool promptly in ice bath to room temperature _____

c. Label bottom of plates prior to adding discs _____

d. Vortex for 10 seconds; use within 3 minutes _____

e. Add 90 µL of heated samples to a disc on plate as in item 9.d _____

2. Confirmation using beta-lactamase **(optional by State Regulatory Agency)** _____

a. Add one beta-lactamase (red) tablet to each of the heated samples and mix samples as in item 9.c.1 _____

b. Let particulates settle for 1 min then add 90 µL to a disc on plate (Avoid clogging pipet tip with particulates by pipetting from top of samples) _____

c. Or, alternatively add 50 µL of beta-lactamase liquid concentrate (item 6.c), mix samples, wait 1 min then add 90 µL to a disc on plate _____

3. Proceed as in items 9.d-l _____

b. Results of Presumptive Positive, Confirmation, and optional beta-lactamase test _____

1. Inhibitor present _____

a. Zones ≥ 16mm of the heat treated (10.a.1) sample is **Positive for inhibitor** _____

2. Beta-lactam present (optional beta-lactamase test) _____

a. A zone around the disc containing the heat treated milk sample (10.a.1) but no zone around the disc containing beta-lactamase (10.a.2), treated milk sample, sample is **Positive for beta-lactam** _____

b. Zones around the heat treated sample (10.a.1) of equal size, or < 4 mm greater, than beta-lactamase treated sample (10.a.2) is **Positive for inhibitor** _____

c. Zones around both the heat treated milk sample disc (10.a.1) **and** the beta-lactamase treated milk sample disc (10.a.2), **and** the zone around the heat treated milk sample disc is at least 4 mm larger than the zone around the beta-lactamase treated milk disc (10.a.1) [ex. beta-lactamase = 14 mm, untreated = 18 mm], sample is **Positive for beta-lactam and non-beta-lactam inhibitor** _____

c. Confirmation of Appendix N samples, see App. N GR from items 12-13, perform confirmation as in item 10 above (use of beta-lactamase required) and interpret as in items 10.b above _____

d. Verification of Initial Positive Tanker (see App. N GR item 11) or Producer (see item App. N GR item 13.c-g). Duplicate samples tested using beta-lactamase specific test kit; conduct test as in respective FORM FDA/NCIMS 2400 for the test kit; if beta-lactam not detected in either sample duplicate, verify sample using the Charm BsDA test kit as described in item 10 above _____

11. Recording and Reporting (for Appendix N also see App. N GR item 14) _____

a. Record numeric values for all measurable zone sizes for samples **and** controls (screen and confirmation), if no zone is observed record as **No Zone (NZ)** _____

b. Report presence of inhibitor only from heated milk samples _____

c. Report sample as **Positive for inhibitor** (if heat only used 10.a.1) or where demonstrated in beta-lactamase test (10.a.2), and zone size ≥16 mm (10.b.1 or 10.b.2.b), **report to State Regulatory Agency** _____

d. Report as **Positive for beta-lactam** where demonstrated in beta-lactamase test (10.a.2) and when zone size ≥16 mm (10.b.2.a); **report to State Regulatory Agency** _____

e. If both beta-lactam and non-beta-lactam inhibitors are demonstrated in beta-lactamase test (10.a.2) and zone size ≥16 mm (10.b.2.c), report test as **Positive for beta-lactam and inhibitor**; **report to State Regulatory Agency** _____

f. Report numeric values for **all** measurable zone sizes for samples **and** controls _____

g. Report when zone size > 12.7 and < 16 mm as positive but Below Actionable Level _____

h. Report absence of inhibitor (no zone) as **Not Found** _____

i. If any inhibitor is present, i.e., zone > 12.7 mm, bacteria counts cannot be reported _____