

CHAPTER 12. EXAMINATION OF MEAT AND POULTRY PRODUCTS FOR *BACILLUS CEREUS*

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12.1 Introduction

Bacillus cereus is one of the few sporeforming, aerobic bacteria recognized as a bacterial pathogen. It is widespread in soil, milk, the surfaces of meat and poultry, cereals, starches, herbs and spices. Its' role as a food-borne pathogen is relatively recent and somewhat uncommon in the United States. Two distinct types of illness have been attributed to the consumption of food contaminated with *B. cereus*. The more common manifestation is a diarrheal illness with an incubation time of 8-16 h characterized by abdominal pain and diarrhea. The other is an emetic illness with an incubation time of 1-5 h and characterized by nausea and vomiting. While the emetic type is usually associated with cereal type products such as rice, the diarrheal type is more widely associated with many foods.

B. cereus typically is a very large, aerobic, Gram positive, sporeforming rod with peritrichous flagella. It grows over a wide temperature range (10 to 48°C) with an optimum range of 28 to 35°C. It will grow over a wide pH range (pH 4.9 - 9.3) and in sodium chloride concentrations approximating 7.5%. Microscopically it may be seen in chains. Macroscopically the colonies have a dull or frosted appearance on a nutrient agar plate. Its association with disease is usually related to counts $>10^5$ cfu/g in the suspect food. Since *B. cereus* does not ferment mannitol, does produce lecithinase and is resistant to polymyxin, a selective medium consisting of mannitol-yolk-polymyxin (MYP) is commonly used for its isolation. Colonies typically are pink in color and surrounded by a zone of precipitate. An ELISA test is available to detect the diarrheal toxin.

12.2 Equipment, Reagents, Media

12.21 Equipment

- a. Balance capable of weighing to 0.1 g
- b. Stomacher™ (model 400 by Tekmar, or comparable model), sterile plastic bags (with twist ties or self-sealing)

- OR blade-type blender, sterile cutting assemblies and blender jars
- c. Sterile supplies, spoons or spatulas, pipettes (1 ml), bent glass rods "hockey sticks", aluminum pie pans (or equivalent)
- d. Incubator, $30 \pm 1^{\circ}\text{C}$
- e. Incubator, $35 \pm 1^{\circ}\text{C}$
- f. Light or Darkfield Microscope
- g. Platinum inoculating loops, 3 mm diameter
- h. Microscope slides and cover slips
- i. Meeker/Bunsen burner with tripod, or hot plate
- j. Pyrex beaker, 250-300 ml size

12.22 Reagents

- a. Butterfield's Phosphate Diluent (BPD) for sample extraction
- b. BPD dilution blanks, 9 ml volume
- c. Basic fuchsin staining solution, 0.5% aqueous

12.23 Media

- a. Plates of Mannitol Yolk Polymyxin (MYP) Agar
- b. Nutrient Agar Slants
- c. BC Motility Medium
- d. Nutrient Agar Plates
- e. Blood Agar Plates, 5% Sheep RBC

12.3 Sampling and Dilution Procedure

- a. Aseptically composite a 25 g or 25 ml sample in sterile bag or blender jar.
- b. Add 225 ml Butterfield's Phosphate Diluent (BPD) to each sample taken.
- c. Stomach or blend for 2 minutes and then prepare serial dilutions of 10^{-2} to 10^{-6} in 9 ml BPD dilution blanks.

12.31 Plating and Examination of Colonies

- a. Pipette 0.1 ml of the homogenate (10^{-1}) and spread it over the entire surface of duplicate, predried MYP plates with a "hockey stick". Repeat the procedure for each of the other dilutions through 10^{-6} . Use a

separate, sterile "hockey stick" for each dilution. Allow the inoculum to dry before incubating the plates.

- b. Incubate all plates in an upright position for 20 to 24 h at 30°C.
- c. After incubation, examine all plates for colonies that are surrounded by a zone of precipitate (lecithinase production) against an eosin pink to lavender agar background (non-fermentation of mannitol). If the areas of lecithinase production coalesce between colonies, look for plates with 10-100 colonies. Count all typical colonies and determine the presumptive count per gram. Remember that the count will be tenfold higher than the dilution, because only 0.1 ml was placed on a plate.

12.32 Confirmatory and Differential Procedures/Tests

- a. Select 4-6 typical colonies for confirmation. Each of these colonies is subcultured on a predried Nutrient Agar Plate and incubated at 30°C for 24 - 48 h. Note the presence or absence of rhizoid growth on the nutrient agar plate.
- b. At the same time inoculate a tryptic soy sheep blood agar plate that has been divided into 4 - 6 segments. A 2 mm loop should be used to deposit the inoculum in the center of the segment. Note the size of the hemolytic zone (and whether it is partial or complete).
- c. Motility test - use BC motility medium method by making a center line stab inoculation with a 3 mm loop and incubating the tube at 30°C for 18-24 h. Observe for diffuse growth into the medium away from the stab as an indication of a motile organism.

Alternatively a microscopic motility test may be used. The slide motility test is done by adding 0.2 ml of sterile water to a nutrient agar slant and then inoculating the aqueous phase with a 3 mm loopful of a 24 h slant culture. Incubate for 6-8 h at 30°C. Place a loopful of the liquid culture on a glass slide and overlay with a cover slip. *B. cereus* and *B. thuringiensis* are actively motile while *B. anthracis* and the rhizoid strains of *B. cereus* are non-motile.

- d. Rhizoid growth - to test for rhizoid growth, inoculate several well isolated areas of a predried Nutrient Agar Plate. Use a 3 mm inoculating loop to make a point of contact inoculation. Incubate the plate in an upright position at 30°C for 24-48 h. If hair-like projections (rhizoids) develop outward from these colonies, the isolate is *B. cereus* var. *mycoides* and not considered to be a human pathogen.
- e. Protein toxin crystal stain - Make a smear on a microscope slide with sterile water from a 2-3 day old nutrient agar plate or slant. Allow the slide to air dry and then gently heat fix it. After cooling, flood the slide with methanol, wait 30 seconds and pour it off. Then flood the slide with 0.5% aqueous solution of basic fuchsin. Gently heat the slide until steam is observed, remove the heat, wait 1-2 minutes and repeat the procedure. Let the slide cool and rinse well with water. Examine under oil immersion for free spores and darkly stained, diamond shaped, toxin crystals. Toxin crystals should be present if the cells have lysed and free spores are observed. The presence of toxin crystals is strongly indicative that the organism is *B. thuringiensis*.
- f. Other Tests - If further biochemical testing is warranted, consult either Bergey's Manual of Systematic Bacteriology or the Compendium of Methods for the Microbiological Examination of Foods.

12.33 Interpretation of Test Results

- a. *B. cereus* usually is: lecithinase positive, strongly hemolytic on sheep blood agar, actively motile, does not produce rhizoid colonies and does not produce protein toxin crystals (diamond shaped).
- b. Other lecithinase positive or weakly positive cultures may be *B. cereus* var. *mycoides*, *B. thuringiensis*, or *B. anthracis*. Caution: non-motile, non-hemolytic colonies could be *B. anthracis* and should be handled with special care.

12.4 Method Quality Control Procedures

A minimum of three method control cultures is recommended for use whenever a new batch of medium is made or acquired as well as each time that an analysis is performed. These controls should consist of at least one strain each of *B. cereus*, *B. cereus* var. *mycoides*, and *B. thuringiensis*. This also will assist the analyst in becoming more familiar with the morphological and cultural differences of these *B. cereus* variants.

12.5 Selected References

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