Presence of Soil-Dwelling Clostridia in Commercial Powdered Infant Formulas

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Objective Because *Clostridium botulinum* was isolated from powdered infant formula (PIF) fed to an infant in the United Kingdom who subsequently developed infant botulism and from unopened PIF from the same manufacturer, we tested PIF manufactured in the United States for the presence of clostridial spores.

Study design Thirty PIF ingested by 19 California infants with botulism within 4 weeks of onset of illness (48% of all patients fed PIF during study) in 2006-2007 were cultured anaerobically to isolate clostridia. All isolated clostridia were identified to the species level and enumerated with standard microbiologic and molecular methods.

Results Five of 30 (17%) PIF samples ingested by patients contained clostridial spores. Spores were also found in 7 of 9 (78%) market-purchased PIF samples. *Clostridium sporogenes* was isolated most frequently, followed by *Clostridium butyricum* and at least 10 other soil-dwelling clostridial species. No neurotoxigenic clostridia were isolated. The most probable number of clostridial spores in PIF ranged between 1.1 to >23 per 100 g.

Conclusions With the notable exception of production of botulinum neurotoxin, *C sporogenes* is physiologically comparable with proteolytic strains of *C botulinum,* and both share the same natural reservoir (soils and dust worldwide). The isolation of *C sporogenes* and potentially pathogenic clostridia from U.S.-manufactured PIF suggests that neurotoxigenic clostridial spores have the potential to be present in these products. *(J Pediatr 2010;156:402-8)*.

Bacteria belonging to the genus *Clostridium* are characterized as anaerobic, endospore-forming Gram-positive bacilli, whose natural habitat chiefly is soil and marine sediments. The clostridia are also found as normal inhabitants of human and animal intestinal flora and excreta.[1-6] Several clostridial species are of medical importance because of the more than 20 protein toxins that they collectively produce.[2-6] These toxins include botulinum and tetanus neurotoxins, respectively, the most potent poisons known, as well as the toxins produced by *Clostridium perfringens* and *Clostridium difficile.*[7] *Clostridium botulinum*, and rarely neurotoxigenic *Clostridium butyricum*[3,7-9] or neurotoxigenic *Clostridium baratii,*[3,7,10,11] can cause infant botulism (IB), the intestinal toxemia form of botulism and the most common form of human botulism in the United States.[12,13]

Honey is the most well-documented food source of *C botulinum* spores for infants and for this reason is universally recognized to be an unsafe food for infants.[14-21] However, in the United Kingdom in 2001 a single case of infant botulism occurred and appeared to be linked to powdered infant formula (PIF) that contained *C botulinum* spores.[22] Laboratory investigations of this case reported in 2005 disclosed that the PIF consumed by the patient contained 4 strains of *C botulinum* type B that could be distinguished by their 4 different amplified fragment length polymorphism (AFLP) patterns and that 2 of these type B strains had colonized the infant.[22] Also, 1 of 2 *C botulinum* type B strains from the patient had a pulsed-field gel electrophoresis (PFGE) pattern that was indistinguishable from the PFGE pattern of 1 of 4 strains isolated from the opened can of formula.[23] In addition, an unopened, factory-sealed container from the same lot of formula that was consumed by the patient yielded another *C botulinum* type B strain with a different PFGE pattern and a fifth AFLP pattern.[22,23]

In light of the United Kingdom experience, we undertook a 2-year surveillance study of U.S.-manufactured PIF consumed by patients hospitalized with IB for the possible presence of *C botulinum* and other clostridial species.

AFLP	Amplified fragment length polymorphism
BoNT	Botulinum neurotoxin
BSM	Botulinum selective medium
CMGS	Chopped meat glucose starch
EYA	Egg yolk agar
FDA	Food and Drug Administration
IB	Infant botulism
MPN	Most probable number
PFGE	Pulsed-field gel electrophoresis
rDNA	Ribosomal deoxyribonucleic acid
SBA	Sheep blood agar

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Methods

Parents of all California infants who have laboratory-confirmed IB are interviewed by our Program to obtain epidemiologic information. Parents of patients who were fed PIF within the 4 weeks preceeding the onset of illness were asked to submit any PIF that remained in the original commercial container from which their child had been fed. Parents were provided with postage-paid shipping supplies and monetary reimbursement for the formula. The study period was January 1, 2005 to December 31, 2006.

PIF fed to patients meeting the study inclusion criteria were submitted directly to our laboratory in the original commercial containers. For laboratory coding purposes, each sample was grouped according to formula brand, which was designated by a roman numeral. Brands I and II accounted for most samples received for testing. Brand I formulas accounted for the greatest number of IB case–associated formulas that were submitted. A smaller number of PIF representing 3 other lesser-used brands were submitted and were assigned to groups III, IV, and V. Additionally, containers of Brand II PIF were randomly purchased from area stores so that more samples of Brand II could be tested and compared with Brand I formulas.

Contamination control

Considerable effort was taken to prevent the possibility of laboratory-introduced spore contamination of the PIF during testing. The work reported was conducted in a closed laboratory room that has extremely low foot-traffic or other movements that would cause any possible dust contaminants to become airborne. Additionally, quality control procedures done monthly to document media sterility determined that no bacterial spore contamination existed within the laboratory. Finally, each time sample testing was undertaken, all work surfaces of the sample testing area, including the edges of overhead shelving, were first decontaminated with a fresh solution of 10% sodium hypochlorite and 70% ethanol.

Presampling preparation and sanitization

The laboratory bench-top where the samples were processed was lined with absorbent plastic-backed paper and soaked with a fresh solution of 10% sodium hypochlorite. A precleaned balance, 4-position magnetic stir plate and all sterile glassware and sterile consumable items were placed on this paper in preparation for sampling. Finally, the formula containers, which were received in clean, zip-sealed, plastic bags, were placed on the decontaminated bench-top. A fresh solution of 10% sodium hypochlorite was used to sanitize all external surfaces of the zip-sealed plastic bags before they were opened to remove the commercial PIF containers. Once removed from the plastic bag, all external formula container surfaces were sanitized with 10% sodium hypochlorite. Extra care was given to sanitizing the plastic cap of previously opened containers or the perforated pop-top seam of factory-sealed containers before these containers were entered for sampling.

Sampling

For factory-sealed containers or for open containers with greater than 100 g of powder, 100.0 g of powder were sampled in 4 portions of 25.0 g each with a sterile, single-use sampling spoon. Each sample portion was transferred to individual, preweighed sterile beakers to which 10 volumes of Butterfield's diluent[24] prewarmed to 50°C was added. Sterile stir bars were added, and the solution was stirred on a magnetic stir-plate until the powder dissolved. The dissolved powder was transferred to individual 500-mL sterile conical centrifuge bottles and spun in a centrifuge at 3450 × g at 4°C for 60 minutes. The supernatant was vacuum-aspirated with sterile glass Pasteur pipettes and discarded; the pellets from the 4 centrifuge bottles were combined and then resuspended in 10 mL of Butterfield's diluent. For previously opened containers with less than 100 g of PIF, all available formula was tested.

Culture and botulinum toxin culture screen

The entire volume of the resuspended pellets (when 100 g was sampled) was equally distributed into a set of 10 culture tubes each containing 20 mL of prereduced chopped meat glucose starch (CMGS) broth. Doing so enabled the calculation of a single 10-tube most probable number (MPN) of spore-forming bacteria contained in the 100.0 g of PIF.[25] When less than 100 g of PIF was sampled, up to 5 tubes of CMGS broth were inoculated. For all samples the inoculated, CMGS broth was heat-shocked in a 70°C water bath for 15 minutes to select for spore-forming bacteria, immediately cooled, and then incubated at 35°C for up to 2 weeks.

CMGS cultures of PIF visually confirmed to have growth at 1 and 2 weeks of incubation were screened for botulinum neurotoxin (BoNT) and also subcultured for isolation and identification of *Clostridium* species. To accomplish these tasks, broth culture was removed from the bottom of each tube and diluted 1:4 in gelatin phosphate diluent. The diluted broth culture was spun in a centrifuge at 3450 *g* at 4°C for 15 minutes. The supernatant was filter sterilized through a 0.45-μm syringe filter and retained for BoNT screening, which was performed with the standard mouse bioassay.[26] The pellet was subcultured onto 2 plates, 4% egg yolk agar (EYA) and botulinum selective medium (BSM),[27] both made with a base of brain-heart infusion. EYA and BSM cultures were incubated either in anaerobe jars or an anaerobe chamber (Sheldon Mfg., Cornelius, Oregon) and incubated for 48 to 72 hours at 35°C. All diluents and media were prepared in-house.

Organism identification

EYA and BSM cultures were examined for colonies exhibiting typical characteristics of the genus *Clostridium*.[2-4,28-30] Suspicious colonies were subcultured to 2 plates of brain-heart infusion agar containing 5% sheep blood (SBA) and to 1 tube of CMGS and then incubated as above. One SBA plate was incubated aerobically to test for the aerotolerant clostridia.[4,28-30] All spore-forming, Gram-positive bacilli that grew anaerobically, or those that were aerotolerant and