UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, DC 20460

OFFICE OF
PREVENTION,
PESTICIDES
AND TOXIC
SUBSTANCES

November 14, 2009

MEMORANDUM

Subject: Protocol Review for Dispatch Hospital Cleaner Disinfectant Towels with Bleach (EPA Reg. No. 56392-8), DP Barcode: 370986

From: Tajah L. Blackburn, Ph.D., Microbiologist
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Product Science Branch
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11/17/09

Thru: Emily Mitchell, Chief
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To: Wanda Henson PM 32
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Applicant: Caltech Industries, Inc.
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I BACKGROUND

The current submission is in response to ongoing difficulties with the ATS C. difficile spore production method and its inability to produce a spore crop with 90% purity as required by EPA. Per the registrant, "Caltech finds it necessary to revise the towelette efficacy testing methodology. We have been working with Dr. Sattar at CREM and have been successful in achieving the 90% spore purity that we could not achieve with the previously reviewed method. I have shared our hurdles with the ATS method and our success with the CREM method with Dr. Tajah Blackburn. As discussed with Dr. Blackburn, we are submitting a revision to the protocol based on CREM's method of

spore production, QCT-2 methodology, and testing of the expressed liquid from the towelette. Though the protocol is the same as was used by CREM to evaluate the currently registered *C. difficile* sporicidal product, we have added the additional controls and method details as requested in the recent EPA protocol responses and as advised by Dr. Blackburn....The wetness testing protocol will remain the same as approved by EPA in the May 2, 2009 Memorandum" (Registrant's letter dated October 15, 2009). The current submission contains a letter from the registrant (as cited above), and a revised protocol for GLP-Compliant Testing. According to the referenced registrant's letter, "the sample label was not resubmitted. Once the protocol is approved, the most current version of the product label will be edited to include the changes requested in the May 2, 2009 Memorandum."

II SYNOPSIS OF REVISED PROTOCOL

Title: Testing Activity of the Expressed Liquid of a Disinfectant Towelette against the Spores of *Clostridium difficile* using the Second Tier of the Quantitative Carrier Test (QCT-2)

Performing Laboratory: Centre for Research on Environmental Microbiology, University of Ottawa

Prepared by: Justo Perez, PhD

Test Organism: *Clostridium difficile* spores (ATCC #43598). For production of high titers of viable spores, the organism will be grown in a 500 ml of a peptone-salts liquid culture medium in 1 L polycarbonate bottles. The exact composition of the culture medium is proprietary to CREM. Briefly, the organism will be inoculated in the growth medium and incubated for five days under strict anaerobic conditions at 36±1°C, the spores collected by centrifugation at 10,000 x g for 10 minutes at 3±1°C, washed three times with a total of 1 L sterile distilled water and enzymatically treated, washed again with about 120 ml of sterile distilled water, heated at 70°C for 10 minutes, suspended in distilled water and stored at 3±1°C. This process can yield suspensions with ≥109 viable spores as colony forming units (CFU)/ml and a purity higher than 90% as determined by spore staining and microscopic examination/counting.

Carriers: Disks of stainless steel (1 cm in diameter; 0.7 mm thick) will be magnetized and brushed stainless steel (AISI type 430). Before use, the new disks will be boiled for 5 minutes in water and thoroughly rinsed with running tap water. They will then be soaked in a 1% solution of 7X detergent for about one hour and rinsed first in tap water, then in distilled water and finally in absolute ethanol. Each disk will be checked for pitting, rust or other damage under a dissecting microscope at a magnification of at least 20X. Disks with any apparent damage or defects will be discarded. The selected disks will be placed in a 20-ml capacity, screw-capped glass vial and sterilized by autoclaving at 121°C for 45 minutes and dried in a drying oven. For inoculation, the required number of disks will be removed from the vial with a pair of sterile [forceps] and placed in a sterile glass Petri dish lined on the inside bottom with filter paper.

Carrier Contamination: The spore suspension will be vortexed three times for 10 seconds each and sonicated for 5 minutes in a sonicator bath just before the inoculation

of the carriers. Ten (10) µl will then be placed on each disk, dried at room temperature (22±2°C) in a laminar flow hood for approximately 60 minutes and then in a dessicator under vacuum for 2 hours at the same temperature. Each carrier must be visually dry before use.

Verification of Test Substance Concentration: Expressed liquid from each lot of test substance will be titrated to confirm the level of available chlorine in parts per million (ppm) as per CREM SOP #DISEXP 005: Briefly, the total (TC) and free chlorine (FC) concentrations will be estimated using the HACH procedure manual for FC (0-200 mg/L) method # 8021 (pages 91-92) and TC (0-200 mg/L) method #8167 (Pages 99-100). Both methods are based on the N-Diethyl-p-phenylenediamine (DPD) indicator reaction. The DPD reagent is ready-to-use in standard pillows supplied for HACH. The three readings will be reported in the final report as an average value. The test samples will be diluted with double-distilled water by adding 0.1 ml in 100 ml final volume. For estimating chlorine concentrations, 0.5 to 1 ml volumes will be diluted to a final volume of 10 ml with double-distilled water. To this volume a DPD pillow will be added, mixed well, and after 3 minutes the concentration in mg/L read in the colorimeter. The measurements will be conducted at least three times on each sample.

Exposure Conditions: Each disk with the dried inoculum will be picked up with a sterile pair of forceps and placed (with the inoculated side facing up) individually in a wide-mouthed Nalgene vial. Fifty (50) µl of the expressed liquid from the test substance will be placed with a pipette on each test carrier; control disks will receive an equivalent volume of PBS + 0.1% Tween-80. The method of expressing liquid will be documented in the raw data and detailed in the final report. The test will be conducted within 3 hours of expressing the liquid. All disks will be held for a contact time to be determined by the Sponsor at room temperature (22±2°C). The timing for the contact time will commence immediately upon the deposition of the test substance or control fluid on the carrier. The carriers will be treated and neutralized in a sequential, timed fashion. Immediately at the end of the contact time, each vial will receive 10 ml of PBS with 0.1% Tween-80 and sodium thiosulfate as the neutralizer (for ease of manipulation, 10 ml will be used instead of 9.95 ml). Each vial will be vortexed for 30 seconds thrice and the eluate subjected to 10-fold serial dilutions in PBS. The number of dilution steps will depend on the expected viable organisms on the test carriers and will be documented in the final report. The relative humidity and air temperature in the room will be monitored using a hygro-thermometer and recorded for each test.

Recovery of the Test Organism: The eluate from each vial will be processed within 30 minutes of the addition of the eluate/neutralizer. Depending on the number of viable organisms expected in the eluate, the undiluted sample as well as its 10-fold dilutions will be separately passed through a 47 mm diameter membrane filter (0.22 µm pore diameter) held in a sterile plastic holder. Each selected 10-fold dilution tube then will be washed three times with 10 ml of PBS, the washes passed through the filters and then each filter holder will be washed at least three times with 10 ml for a total of about 60 ml of PBS (pH 7.2-7.4) (such as washing step is meant to further remover any residues of the test substance on the filters). The filters will then be removed with a sterile pair of forceps and placed on the BHIYT agar Petri plates (BHI, 3.7%; Yeast Extract, 0.5%; L-Cysteine, 0.1%; sodium taurocholate, 0.1%; Lysozyme, 0.5 mg%; Agar 1.5%) and incubated in an anaerobic chamber for up to five days at 36±1°C.

Incubation/Observation: The plates of the recovery medium will be examined for colonies first at the end of 48 hours and then at the end of five days of incubation. The colonies will be counted and their numbers recorded. Representative plates showing survivors will be assayed to confirm the identity of the test organism as detailed below under 'Purity Control'.

Controls:

Spore Purity Control: An isolated colony will be picked up, streaked on FAA plates and incubated anaerobically for 24-48 hours. The culture must exhibit pure colonies matching the following description:

> Odor: A characteristics horse-like smell should be present
> Colony characteristics: Flat, yellow-to-brown colonies with a ground glass-like appearance and a slightly filamentous edge.
> Fluorescence under long wave UV light (~300 -330 nm)—a yellow pale fluorescence should be seen

The spore suspension will be also evaluated for quality by spore staining with malachite green (spores stain green) and counterstaining with Safranin (vegetative cells stain red). A minimum of 5 fields will be examined microscopically to determine the ratio of spores to vegetative cells. The percentage of spores must be ≥90% to confirm the purity of the spore suspension. If this value is not achieved, additional steps will be taken to further concentrate/purify the preparation. Any such additional steps will be detailed in the final report.

Spore purity will be calculated as follows:

=100 X ((avg. number of spores)/ (avg. of cells + average of spores))

Carrier Sterility Control: For each batch of sterilized carrier, one carrier will be removed, eluted in 10 ml of PBS and the eluate membrane filtered and the membrane laid on BHIYT agar. The plates will be incubated anaerobically for 5 days. The acceptance criterion for this control is no growth.

Initial Suspension Population Control: The prepared spore suspension will be appropriately serially diluted in PBS and 0.1 ml aliquots of the 10-4, 10-5, and 10-6 dilutions will be plated on BHIYT agar plates and incubated for 48±4 hours at 36±1°C under anaerobic conditions. Following incubation, the culture plates will be observed to enumerate CFU of the test organism inoculated onto test carriers.

Medium Sterility Control: For each microbiocide test, two BHIYT plates, two BHIYT plates with a sterile membrane filter on them, two BHIYT plates with membrane filters through which 20 ml of PBS has been passed will be incubated anaerobically at 36±1°C for 5 days. The acceptance criterion for this control is no growth.

Neutralization Confirmation Control: It is essential to first validate that the microbiocide neutralization procedure is indeed able to achieve the desired result of arresting activity of the active(s) in the test formulation(s). Such validation of the neutralization will be conducted with each lot of the test substance prior to any testing for sporicidal activity. This will be achieved as follows:

A suspension of the spores will be prepared by diluting the stock in PBS to give colonies in the range of 500-1000 CFU/ml. The diluted microbial suspension (0.1 ml) will be added to 10 ml of (a) PBS, (b) neutralizer alone, and (c) neutralizer containing 50µl of test substance. (Based upon weights obtained during research, the spray treatment imparts approximately 10 µl of product to the carrier thus the use of 50 µl is a stringent representation of the potential volume of residual test substance). The samples will be held for 30 minutes under ambient conditions and membrane-filtered separately. Each filter will be washed with about 60 ml of PBS. The filters will then be placed on plates of BHIYT medium and incubated anaerobically at 36±1°C for 5 days. Colonies will be counted for each control carrier and averaged.

Neutralization will be considered to be validated if the CFU on all plates are comparable to those on the controls in the range of ±15% or less.

Carrier Population Control: Each control carrier must yield enough CFU to indicate the presence of $\geq 10^6$ viable spores. This is essential to meet the product performance criterion of a spore kill of $\geq 10^6$. The spores in the dried inoculum on each control carrier will be exposed to the same volume as in the control fluid and held for the required contact time and eluated. The eluates will be diluted over a range of 5 10-fold dilutions, the dilutions filtered and CFU in them counted.

Study Acceptance Criteria:

Test Substance Performance Criteria: The test substance must meet the EPA requirements of ≥6 log10 or 99.9999% reduction in CFU of the test organism as compared to the carrier population control.

Control Acceptance Criteria: The study controls must perform according to the criteria detailed in the study controls description section.

Data Analysis

Calculations

CFU/ml (sheet attached)

Log reduction

Log reduction= log (Average CFU in the control)-log (average CFU in treated samples)

Percent reduction

Percent reduction= ((CFU in the control –CFU Recovery in treated sample)/ (CFU in the control)) * 100

Statistical Analysis

All data will be entered using Excel program and the same program will be used for data analyses and reporting.

Quality Assurance: The Sponsor will provide an EPA GLP Quality Assurance Unit for the study. This contractor will be responsible for QAU activities for this study including protocol, raw data, SOPs, final report, and in-phase audits, reports to management and the QAU Statement. The study will be conducted in accordance with EPA Good Laboratory Practices (40 CFR 160). Exceptions in compliance will be itemized in the final report.

III CONCLUSIONS

Due to issue regarding spore quantity, the registrant has requested to modify the methodology from AOAC Germicidal Spray Test modified for towelettes to the QCT-2 testing the expressed liquid. The testing of expressed liquid using to the QCT-2 is acceptable to support towelette registration against *Clostridium difficile* spores. The carrier control population must be $>10^6$, not $\geq 10^6$. The proposed testing lab is not GLP approved. To address this issue the registrant included in the current protocol the following explanation: "The Sponsor will provide an EPA GLP Quality Assurance Unit for the study. This contractor will be responsible for QAU activities for this study including protocol, raw data, SOPs, final report, and in-phase audits, report to management, and the QAU Statement. The study report will be conducted in accordance with EPA Good Laboratory Practices (40 CFR 160). Exceptions in compliance will be itemized in the final report. A label was not included in the current submission." Furthermore, the wetness determination, as accepted by the Agency, is still required to support *C. difficile* towelette claims.