Production and Validation of the Use of Gamma Phage for Identification of Bacillus anthracis

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Gamma phage specifically lysed vegetative cells of Bacillus anthracis and serves as part of the basis for identification of isolates from agar cultures. We report our study to standardize gamma phage production and preparation and to validate the assay for routine use. Unstable phage preparations were largely reduced through propagation of phage on blood agar cultures of the avirulent B. anthracis strain CDC684 and were adequately stable for extended storage beyond 1 to 2 years at 4°C, provided that the preparation initially gave rise to clearly discernible plaques (macroplaques, 5 to 10 mm in diameter) on dilution at 1:8 or greater during potency testing with the Sterne strain or its equivalent. The primary intent of the assay was to test nonhemolytic, ground-glass-appearing bacterial B. anthracis-like colonies arising from culture of clinical or nonclinical samples on 5% sheep blood agar. Specifically, the assay was designed to show clear or primarily clear circular zones of lysis on bacterial lawns at the site of gamma phage inoculation after incubation at 35 °C ± 2°C for 20 h. When tested with 51 B. anthracis strains and 49 similar non-B. anthracis Bacillus species, the analytical specificity was >95%, a value that is intentionally low because our study design included two rare nonsusceptible B. anthracis strains as well as a rare susceptible non-B. anthracis strain, B. cereus ATCC 4342. Repeatability, day-to-day precision, and analyst-to-analyst precision were superior. The assay was rugged to variations among phage lots, phage concentration, amounts of bacterial inoculum, and incubation times as short as 6 to 8 h. System suitability evaluation showed improved robustness when bacterial lawns were tested with high-and low-density inoculum using the first and second quadrants of a serial four-quadrant streak on 5% sheep blood agar plates.

Bacteriophages that are active on B. anthracis but not specific were first described in 1931 by Cowles (7). In 1951, McCloy isolated a bacteriophage called “phage W” from what she described as an atypical Bacillus cereus strain, designated strain W, ATCC 11950 (9). The phage was highly specific for 171 strains of B. anthracis and failed to react with 19 other Bacillus species, except for two unusual strains of B. cereus, strains NCTC 1651 and NCTC 6222 (ATCC 4342). In a subsequent study, McCloy (10) isolated two types of phage from aging cultures of Bacillus strain W, referred to as “phage α” (rare mutant) and “phage β” (dominant form), which specifically produced plaques on agar cultures of B. anthracis. The two phages differed only in that phage α attacked strains of B. anthracis and Bacillus strain W. Phage β attacked all B. anthracis strains tested by McCloy but not strain W; however, it was not able to attack smooth or encapsulated forms of B. anthracis. In 1955, Brown and Cherry (4) isolated a variant of the original phage W, designated gamma phage. This phage differed from phage α and phage β in that it lysed encapsulated smooth forms of B. anthracis, failed to lyse any of the strains of B. cereus tested, and could lyse and be propagated on the Bacillus W strain. Use of gamma phage as a diagnostic tool for identification of B. anthracis has been cited in a number of articles (2–5, 12). Other sensitive Bacillus species included a soil-derived B. megaterium strain at Kansas State University (6) and four nonrhizoid strains of B. cereus var. mycoides (5). Buck et al. (6) reported these two strains to be negative in the “string-of-pearls” assay, which is typically positive for B. anthracis strains. In contrast, several purported B. anthracis isolates have been reported as not susceptible to gamma phage through either the inability of phage to bind or the specific lytic action of the PlyG lysin enzyme (11) isolated from gamma phage.

The goals of this study were to document and validate the performance characteristics of the gamma phage lysis assay for B. anthracis identification, standardize gamma phage production methods, and determine stability. The validation of the assay considered the following analytical performance parameters as listed and defined in the United States Pharmacopeia, section 1225, as appropriate: precision, accuracy, selectivity/specificity, quantification limit, detection limit, linearity, and range (13). To establish a series of system suitability parameters, elements of precision and robustness were considered. Under criteria in United States Pharmacopeia, section 1225 (10th Supplement), this procedure falls within the category of an identification test (category IV). Thus, specificity is the most important performance characteristic. Elements of accuracy, quantification limit, detection limit, linearity, and range were not considered because this is a qualitative test.

MATERIALS AND METHODS

Gamma phage and Bacillus strains. Gamma phage was originally obtained from the Centers for Disease Control and Prevention (CDC), Atlanta, Ga.,...
### Production and validation of the use of gamma phage for identification of Bacillus anthracis, Journal of Clinical Microbiology 43:4780 - 4788

**Gamma phage specifically lyses vegetative cells of Bacillus anthracis and serves as part of the basis for identification of isolates from agar cultures.** We report our study to standardize gamma phage production and preparation and to validate the assay for routine use. Unstable phage preparations were largely reduced through propagation of phage on blood agar cultures of the avirulent B. anthracis strain CDC684 and were adequately stable for extended storage beyond 1 to 2 years at 4 degrees C, provided that the preparation initially gave rise to clearly discernible plaques (macroplaques, 5 to 10 mm in diameter) on dilution at 1:8 or greater during potency testing with the Sterne strain or its equivalent. The primary intent of the assay was to test nonhemolytic, ground-glass-appearing bacterial B. anthracis-like colonies arising from culture of clinical or nonclinical samples on 5% sheep blood agar. Specifically, the assay was designed to show clear or primarily clear circular zones of lysis on bacterial lawns at the site of gamma phage inoculation after incubation at 35 degrees C +/- 2 degrees C for 20 h. When tested with 51 B. anthracis strains and 49 similar non-B. anthracis Bacillus species, the analytical specificity was >95%, a value that is intentionally low because our study design included two rare nonsusceptible B. anthracis strains as well as a rare susceptible non-B. anthracis strain, B. cereus ATCC 4342. Repeatability, day-to-day precision, and analyst-to-analyst precision were superior. The assay was rugged to variations among phage lots, phage concentration, amounts of bacterial inoculum, and incubation times as short as 6 to 8 h. System suitability evaluation showed improved robustness when bacterial lawns were tested with high- and low-density inoculum using the first and second quadrants of a serial four-quadrant streak on 5% sheep blood agar plates.

**Subject Terms:**

Bacillus anthracis, anthrax, gamma phage, identification, assay
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Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18
Organism and strain (origin) | Organism and strain (origin)
---|---
**B. anthracis** strains | **B. mycoides** ATCC 10206
**B. anthracis** (resistant) | **B. cereus** NRS 820
**B. anthracis** C-8800C | **B. mycoides** ATCC 6562
**B. anthracis** 57 | **B. licheniformis** ATCC 12759
**B. anthracis** Albia (Iowa) | **B. mycoides** ATCC 31101
**B. anthracis** New Hampshire | **B. licheniformis** ATCC 14580
**B. anthracis** Pen. Res. 22-5-85 | **B. subtilis** PA2
**B. anthracis** ATCC 4127 | **subtilis** PY143
**B. anthracis** Buffalo | **polymyxa** ATCC 842
**B. anthracis** Nebraska | **bustis** ST-1
**B. anthracis** Ames | **mycoides** ATCC 1664
**B. anthracis** SK-61 | **licheniformis** ATCC 6634
**B. anthracis** M | **bustis** BGSC1A2
**B. anthracis** Colorado | **sphaerica**
**B. anthracis** Vollaum 1B | **thuringiensis** ATCC 10792
**B. anthracis** SK-102 | **amyloidogalacsiens**
**B. anthracis** LA-1 | **mycoides** (B. anthracis) CDC680
**B. anthracis** Scotland | **bustis** BGSC 3A1
**B. anthracis** 1928 (cow, Iowa) | **licheniformis** ATCC 12173
**B. anthracis** (resistant) | **bustis** BP-1
**B. anthracis** E87-3-21, R3 | **mycoides** ATCC 9954A
**B. anthracis** delta-Ames | **cereus** ATCC 9620
**B. anthracis** CDC673 (England) | **cereus** ATCC 7064
**B. anthracis** CDC572 (Argentina) | **pyrocumulum** BGSC 7A2
**B. anthracis** Okonedka | **megaterium** BGSC 7A1
**B. anthracis** G-28 | **megaterium** ATCC 14581
**B. anthracis** VLA-1770 | **bustis** CDC678
**B. anthracis** CDC608 (Canada) | **bustis** CDC693/NRS 744
**B. anthracis** SK-128 | **bustis** BGSC
**B. anthracis** Sterne | **licheniformis** ATCC 9859
**B. anthracis** LA-2 (phage sensitive) | **medusas** Delaporte ATCC 25621
**B. anthracis** CDC77 (Pakistan) | **bustis** W-23 ATCC 23059
**B. anthracis** V770-NP1-R | **cereus** ATCC 4342
**B. anthracis** V770 | **cereus** ATCC 11950
**B. anthracis** SK-465 | **cereus** ATCC 9634
**B. anthracis** SK-31 (South Africa) | **bustis** BST-2
**B. anthracis** SK-162 (Haiti) | **bustis** niger (in house)
**B. anthracis** Pen. Res. 9-12-83 | **mycoides** ATCC 21929
**B. anthracis** ZA-28-5-85 | **mycoides** ATCC 22558
**B. anthracis** VH | **licheniformis** ATCC 6563
**B. anthracis** CDC476 (Pakistan) | **licheniformis** ATCC 12759
**B. anthracis** Arkansas | **cereus** ATCC 15817
**B. anthracis** delta-ANR | **thuringiensis** ATCC 4040
**B. anthracis** ZIM 89 | **thuringiensis** ATCC 4041
**B. anthracis** Minnesota | **mycoides** ATCC 10206
**B. anthracis** 002BSI | **licheniformis** ATCC 14580
**B. anthracis** LA2 isolate 2 | **thuringiensis** ATCC 4040
**B. anthracis** LA2 isolate 3 | **mycoides** ATCC 10205
**B. anthracis** Weybridge Sterne A | **licheniformis** ATCC 14580
**B. anthracis** CDC607

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*Non-Bacillus anthracis* strains:

- **B. mycoides** ATCC 10206
- **B. cereus** NRS 820
- **B. mycoides** ATCC 6562
- **B. licheniformis** ATCC 12759
- **B. mycoides** ATCC 31101
- **B. licheniformis** ATCC 14580
- **B. subtilis** PA2
- **subtilis** PY143
- **polymyxa** ATCC 842
- **bustis** ST-1
- **mycoides** ATCC 1664
- **licheniformis** ATCC 6634
- **bustis** BGSC1A2
- **sphaerica**
- **thuringiensis** ATCC 10792
- **amyloidogalacsiens**
- **mycoides** (B. anthracis) CDC680
- **bustis** BGSC 3A1
- **licheniformis** ATCC 12173
- **bustis** BP-1
- **mycoides** ATCC 9954A
- **cereus** ATCC 9620
- **cereus** ATCC 7064
- **pyrocumulum** BGSC 7A2
- **megaterium** BGSC 7A1
- **megaterium** ATCC 14581
- **bustis** CDC678
- **bustis** CDC693/NRS 744
- **bustis** BGSC
- **licheniformis** ATCC 9859
- **medusas** Delaporte ATCC 25621
- **bustis** W-23 ATCC 23059
- **cereus** ATCC 4342
- **cereus** ATCC 11950
- **cereus** ATCC 9634
- **bustis** BST-2
- **bustis** niger (in house)
- **mycoides** ATCC 21929
- **mycoides** ATCC 22558
- **licheniformis** ATCC 6563
- **licheniformis** ATCC 12759
- **cereus** ATCC 15817
- **thuringiensis** ATCC 4040
- **thuringiensis** ATCC 4041
- **mycoides** ATCC 10205

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*Note:* The table includes a list of test organisms for specificity determination. The entries are categorized based on species and strains, with some notable organisms like **B. anthracis** and **B. mycoides** being highlighted. The table also mentions the use of gamma phage and its application in the context of identifying these organisms. The text also refers to various phage preparations and their use in detecting specific strains, emphasizing the importance of specificity in identifying anthrax and related species. The data reflects the collaborative effort involving experts from different institutions, highlighting the comprehensive approach in scientific research. The text indicates the use of gamma phage in distinguishing between related and non-related strains, which is crucial for understanding the epidemiology and classification of Bacillus species.
determined using the last dilution of gamma phage which gave rise to a distinct macroplaque (5 to 10 mm in diameter), determined to be 1:16 for the Ames strain and 1:16,384 for the Pasteur strain (Fig. 1). The small microplaques (1 to 2 μm) within the site of application of higher dilutions permitted enumeration. As seen in Fig. 1 (bottom, right), for the Pasteur strain (see arrow), microplaques were counted, and the dilution factor was used to calculate the PFU per milliliter. For example, in Fig. 1, the gamma phage underwent 19 twofold dilutions for a final dilution of 1:262,144. In that dilution, 5 μl (1/200 of a milliliter) was spotted and four microplaques were counted in the last area of application (Fig. 1, arrow), and the PFU per milliliter was determined by an algorithm (4 PFU x 200 ml^-1 x 262,144) to be 2.1 x 10^8 PFU/ml.

**Determination of the concentration by pour plate.** The PFU per milliliter were alternatively determined by serially diluting phage 10-fold and incubating phage in the presence of *B. anthracis* in semisolid LB top agar (1). Growth of *B. anthracis* Pasteur vaccine strain cultures, incubated 18 to 20 h at 35°C on SBA, was used to prepare a suspension comparable to a McFarland standard of 0.5 at 600 nm in PBS using a spectrophotometer. Aliquots (1.35 ml) of this suspension were prepared for each 10-fold dilution of phage (10^{-1} to 10^{-6}) diluted in nutrient broth. Starting with the phage dilution of 10^{-3}, 0.15 ml was transferred to each tube in a triplicate set of 1.35-ml cell suspensions, gently subjected to vortex mixing, and then allowed to react for 10 min at 35 ± 2°C. A total of 0.4 ml of phage/bacterial suspension was added to 9.6 ml of melted overlay agar (held at 53°C), gently mixed by inverting the tube several times, immediately poured into a sterile petri dish, covered, and allowed to solidify at room temperature. The process was repeated twice for each phage dilution to be tested. Control plates consisting of *B. anthracis* Pasteur strain devoid of phage were prepared. Cultures were then inverted and incubated at 35 ± 2°C. For each dilution, the presence of distinct phage microplaques was determined, and the total number of plaques was counted in the agar using a dilution plate that yielded 30 to 300 plaques (Fig. 2). The total PFU were calculated in the phage preparation.

**Gamma phage lysis assay.** Culture isolates to be tested for gamma phage sensitivity were pure cultures or had well-defined single colonies in a mixed bacterial population. If culture integrity with respect to age or purity was in doubt, the culture was subcultured to produce isolated colonies on 5% SBA. Suspect colonies selected for testing were nonhemolytic, opaque, slightly raised, irregular (although round colonies can form) with serrated edges, and gray-white with a ground-glass appearance. Suspect colonies typically showed tenacity when the colony was probed with an inoculation loop or needle and “whipped up.” Although the assay was only validated here using vegetative culture growth from SBA cultures, spore suspensions with adequate concentration to yield confluent lawns could also be tested directly and backed up with the validated process (unpublished observation). Positive and negative control cultures were tested concomitantly. Inoculation of test samples and controls was standardized using a 1-μl loop, with which sufficient culture growth was removed to make an approximate 1-mm bead of cells, preferably from an individual colony. The growth was transferred to a fresh SBA plate by streaking a vertical line from the edge towards the center (approximately 1 in. in length) in the first quadrant. The inoculum was spread in close horizontal streaks across the vertical streak site in the first quadrant. Use of the flat side of a 10-μl disposable loop facilitated creation of a uniform inoculation. Using the same loop, several light sweeps were made across one end or edge of the first quadrant, and the close horizontal spreading was repeated in the second quadrant of the plate. This was continued as a streak for isolation in the remaining quadrants as a test of culture purity. Gamma phage suspension (5 μl) was placed on the agar surface in centers of both the first and second quadrants for each test and control plate (as shown in Fig. 3) using a clean micropipettor tip for each application. After replacing the plate lid, circles were drawn on the lid above the sites where phage was applied. The sides of the plate lid and bottom were marked to allow for realignment of the top and bottom before the plates were read postincubation. Agar cultures were incubated at 35°C ± 2°C for 20 ± 4 h. Acceptance criteria for positive assay results were that there must be a clear zone (macroplaque approximately 5 to 10 mm in diameter) of no growth where phage was applied to the positive control in either the first or second quadrant. (Note that it is possible for a few colonies to emerge within the clear zone on the positive control.) A lawn of confluent growth must be present in the first or second quadrants on all culture plates (controls and test unknowns), and quadrants 3 and 4 were inspected to confirm purity. A positive test yielded plaque formation (usually 5 to 10 mm in diameter) at the point of gamma phage application after 20 ± 4 h incubation. In practice, plaques are often seen in 4 to 8 h against the agar surface dulled by early bacterial growth around the site of gamma phage application.

**Protocol, procedure documentation, and training.** The study was described prospectively by USAMRIID validation protocol (VP012). The test method was defined by a study-specific procedure (AN-D9-01) that was used throughout the study. Four analysts participated in the study and were identified as analysts A through D. Results were recorded on standard study data forms. Personnel were trained in the test method, were familiar with good laboratory practices, and were certified for biosafety level 3 laboratory operations.

Culture observations and gamma phage plaque formation were recorded on all plates by digital photography with a Kodak DC290 digital camera. All plates...
were photographed within 36 h of the end of the test. Pixel resolution was set to medium (1,140 × 960 = 1.382 megapixels), and picture format was uncompressed (TIFF). The camera was set to record date/time on the image and watermark text indicating the protocol number. Images were numbered with Kodak’s absolute numbering method. Memory card number, image name, camera settings, and comments were recorded on data sheets. Image data were uploaded from compact flash cards through a personal computer and directly downloaded to a read-only compact disk. Data files were also backed up to an Iomega Zip Drive in picture albums (folders) as captured to the card.

**Equipment and reference materials.** The bacteriological incubator, biological safety cabinets, and micropipettes were calibrated and maintained by the USAMRIID Medical Maintenance Branch and monitored by users. Digital camera and compact flash cards were used and monitored by the study director (J.E.B.).

**Archiving.** Gamma phage and positive and negative control strains along with *Bacillus* species used for specificity testing and testing of precision parameters are maintained as part of the USAMRIID Diagnostic Systems Division bacterial reference library collection.

**RESULTS**

Selection of the gamma phage method as an identification method for *B. anthracis* required documentation of the performance characteristics of the assay. The method was assessed for sensitivity and specificity, precision parameters, and various elements of robustness.

**Variation in gamma phage susceptibility.** A key finding of this study was that various *B. anthracis* strains may differ in their response to gamma phage and their sensitivity to bacterial inoculum level. Representative results are shown in Fig. 3. Susceptibility, as assessed by plaque formation, can vary as a function of the strain and plasmid profile. Fully virulent strains possessing both virulence plasmids tended to produce more clearly defined plaques in the second quadrant than in the first as shown in Fig. 3b to d in that they appeared to be more sensitive to inoculum overload. However, avirulent strains lacking the pXO1 toxin plasmid (i.e., Pasteur strain) and delta-Ames routinely produced large, clearly defined plaques in both quadrants as shown in Fig. 3e and f. The Sterne strain, which lacks the pXO2 capsule-encoding plasmid but possesses the toxin-encoding pXO1 plasmid, formed more clearly defined plaques in the first quadrant. As previously stated, *B. anthracis* Pasteur (Fig. 3f) and *B. cereus* NCTC 2599 (Fig. 3g) served as positive and negative controls throughout the study.

**Potency and determination of PFU per milliliter of gamma phage preparations.** The previously mentioned variations in gamma phage susceptibility of *Bacillus* strains led to variations in determining the PFU per milliliter and potency of phage preparations. *B. anthracis* strains lacking the pXO1 plasmid (i.e., Pasteur and delta-Ames [Fig. 3e and f]) were more susceptible to the gamma phage than those possessing the plasmid (i.e., Sterne, Ames, etc. [Fig. 3a to d]). As a result, only strains lacking the pXO1 plasmid could be used for determining PFU per milliliter. When higher dilutions of phage were plated on the Pasteur strain, individual microplaques were readily detected as small 1- to 2-mm clear zones against confluent bacterial growth (Fig. 1, arrows); however, such plaques were not well formed with the Ames or Sterne strain. The use of the Pasteur strain allowed counting of the PFU per milliliter in preparations held over long periods of storage at 4 ± 2°C. The concentration of phage lot TA101201 was prepared in October 2001 and was tested in February 2002, 2003, and 2004 and found to contain 7.5 × 10^8, 3.0 × 10^8, and 3.23 × 10^8 PFU/ml, respectively, by the pour-plate technique. Likewise, phage lot TA022303, prepared in February 2003, had a concentration of 1.4 × 10^9 PFU/ml, and in February 2004, the concentration dropped to only 3.8 × 10^8 PFU/ml. As shown in Fig. 4, the PFU per milliliter determined using the potency assay was 2.9 × 10^10 PFU/ml on February 2003 and dropped to 7 × 10^9 PFU/ml after a year. It is clear that there was less than a log_{10} loss in activity over 1 year of storage. As for potency determination, in which serial dilutions of phage were placed on a

![Fig. 2. Pour-plate plaque counts using *B. anthracis* strain Pasteur. The example shows 10^{-5} and 10^{-6} dilutions in pour plates.](image)
confluent inoculum of *B. anthracis* Sterne or Ames, a much higher concentration of gamma phage was required to generate macroplaques than that of Pasteur, which formed macroplaques and, ultimately, microplaques at much higher dilutions (Fig. 2 and 4). Using the potency assay, the counts for microplaques were typically higher than those determined using the pour-plate technique, a phenomenon which is not understood. In practice, the gamma phage potency of production lots was assessed using either the Ames or Sterne strain in combination with the Pasteur strain of *B. anthracis*. For laboratories acquiring gamma phage from outside sources, the potency test may be implemented as part of the laboratory quality control for monitoring gamma phage during long-term storage at 4°C. At a minimum, the Sterne strain or its equivalent should be used as a positive control since it more readily reflects the characteristics of fully virulent strains such as Ames.

**Demonstration of precision parameters.** Repeatability was examined by testing multiple (*n* = 5) replicate tests of five independent *B. anthracis* strains (Table 2, analyst A, test 1). In the second quadrant, the test was 100% positive for all five test strains, whereas results were variable in the first quadrant where bacterial lawns were heaviest. Two strains, delta-Ames and Vollum 1B, tested consistently positive, but the Sterne and Ames strains tested consistently false negative in the first quadrant. Overall, first-quadrant results were 56% (14/25) positive for this test of intra-assay variability.

Several components of intermediate precision were tested, such as day-to-day variability, analyst-to-analyst variability, and phage lot-to-lot variability. For day-to-day variability, tests performed by an analyst were 100% positive in the second quadrant over 3 days (Table 2, analyst A, tests 1, 2, and 3). In the first quadrant, results for tests 2 and 3 were similar to those seen in test 1, with 60% and 48% of plates positive. Over time, delta-Ames and Vollum 1B strains were consistently most sensitive in this quadrant, whereas Sterne and Ames strains were consistently among the least sensitive. These strains appeared to have heavy growth in the first quadrant. Comparison of results among three analysts showed similar patterns (Table 2, test 1 for analysts A, B, and C). All tests were 100% positive in the second quadrant, whereas first-quadrant results were 56%, 64%, and 56%, respectively, for the three analysts. Between analysts, the Sterne strain tested inconsistently in the first quadrant. Table 3 shows results of phage lot-to-lot variability using the Sterne strain as a test strain. In the second quadrant, the test was 100% positive for the three lots tested, and no lot provided acceptable results for the first quadrant (23% positive).

**Demonstration of robustness parameters.** Three parameters of assay robustness were evaluated: phage concentration, inoculum size, and incubation time, using the Ames strain as the test strain for all tests. The effect of phage concentration was evaluated as a measure of phage activity or potency by comparing undiluted phage preparations to preparations diluted to 90%, 80%, 70%, and 60% of the original concentration (Table 4). All tests were acceptable at up to a 40% dilution (i.e., 60% of the original concentration) for second-quadrant observations. Again, first-quadrant results were 20 to 40% positive, with no observable trend. This is consistent with the findings that gamma phage preparations can be highly diluted and still form macroplaques (Fig. 1 and 4). When different bacterial inoculum quantities were compared, the data demonstrated that quantity, within the range tested, did not matter (Table 5). Inoculum volumes were approximately 1-, 2-, and 3-mm-diameter beads of inoculum. At all three levels, the assay was 100% positive in the second quadrant but 28% (6/21) positive under heavier bacterial lawn conditions in the first quadrant. The variation in macroplaque formation was shown in Fig. 3. Finally, the method was assessed after incubation for 8 to 24 h. The assay was acceptable after incubation periods as short as 8 h (Table 6). At 16 h, four of five replicates were positive;
however, the one negative result at 16 h for the second quad-
rant was positive in the plate’s first quadrant, nevertheless, 
giving an overall positive observation.

**Demonstration of specificity.** Species sensitivity and speci-
ficity were considered the most important performance param-
eters of this assay. The assay was used to screen 50 indepen-
dent, known strains of *B. anthracis* and 50 strains of non-*B.
anthracis* Bacillus species. We intentionally included two non-
susceptible strains in the *B. anthracis* panel and two sensitive 
non-*B. anthracis* Bacillus strains in the non-*B. anthracis* panel 
(Table 1). Results initially appeared to correspond exactly with 
expected outcomes. However, we report 96% positive specific-
ity for *B. anthracis* reflecting that two known nonsusceptible 
strains were not positive (Table 7). First-quadrant results were 
72% positive. For the non-*B. anthracis* Bacillus strains, there 
were 48 of 50 strains that tested negative, demonstrating a 
specificity of at least 96%. However, in subsequent analysis, 
one of the two positive strains previously catalogued as a *B.
mycoides* strain (CDC680) was found by molecular methods 
after this study to be a *B. anthracis* isolate devoid of both 
plasmids. Further analysis of strain CDC680 by Tetracore, Inc., 
Gaithersburg, Md., using their Red Line assay, revealed that 
the strain tested positive for *B. anthracis*. This identity was 
confirmed by Paul Jackson, Los Alamos National Laborato-
ries, Los Alamos, N.M., and tested positive by PCR at 
USAMRIID for *B. anthracis*-specific chromosomal markers 
but was negative for both virulence plasmids pXO1 and PXO2 
(personal communication with D. Norwood and M. Frye, 
USAMRIID). *B. cereus* ATCC 4342 was negative for chromo-
somal, pX01, and pX02 PCR targets, whereas the two *B. an-

![Image](https://via.placeholder.com/150)

**TABLE 2.** Precision within assay, between days, and between analysts for identification of *B. anthracis* with gamma phage lysis assay

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<tr>
<td>Total</td>
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<td>14</td>
</tr>
</tbody>
</table>

<sup>a</sup> Defined as positive in either the first or second quadrant.

<sup>b</sup> Test 1.
thracis strains that were resistant to gamma phage were positive for the B. anthracis-specific chromosomal target sequence and for pXO1. Additionally, one of the isolates was positive for pXO2, and the other was negative. For this strain, there was no observed lysis in the second quadrant, whereas it was positive in the first quadrant. Recalculated results indicate 98% (48/49) negative specificity for non-B. anthracis strains. Similar results were obtained when plates were read independently by a second reader (analyst D) on the same day (Table 7). Identities of the resistant B. anthracis strains are withheld from open publication. Information on the identity of these nonsusceptible strains may be obtained by authorized persons upon request (Commander, USAMRIID, 1425 Porter Street, Fort Detrick, MD 21702-5011).

Systems suitability considerations: The data demonstrate a tendency for analysts to streak too heavily when spreading a bacterial inoculum. This behavior is noticeable when one compares the difference in response rates between the first and second quadrants. In many circumstances, first-quadrant lawns were too heavily streaked to observe phage lysis. On average, first-quadrant results were not acceptable when considered alone at any time point. The assay reliability depended on observation of second-quadrant results. Analysts should ensure that bacterial lawns are not streaked too heavily in the first quadrant. In contrast, some strains of B. anthracis may not grow to a confluent lawn in the second quadrant under assay conditions. For these isolates, testing phage sensitivity in the first quadrant may provide a satisfactory alternative.

B. anthracis Pasteur proved to be an excellent positive control strain. It was 100% positive in both the first and second quadrants of the assay throughout the study. B. cereus was 100% negative for sensitivity to phage over the course of the study and was therefore an excellent negative control strain. It was 100% positive in both the first and second quadrants. Recalculated results indicate 98% (48/49) sensitivity to phage over the course of the study and was therefore an excellent negative control strain. It was 100% positive in both the first and second quadrants.

### DISCUSSION

The classical gamma phage identification assay for B. anthracis was standardized and evaluated for routine test laboratory use. The acceptance criteria set forth in validation protocol USAMRIID VP012 required at least an 80% expected response for precision and robustness characteristics. For sensitivity and specificity, acceptance criteria were 90% of the expected response, a response set high to reflect the importance of correctly identifying tested isolates. The data in Tables 2 to 7 show that the acceptance criteria were met or exceeded. The assay exhibited a high level of precision for identical strains and between strains on the same day of testing. It displayed high reproducibility for day-to-day and analyst-to-analyst comparisons (Table 2) and produced high precision between the three lots of phage tested (Table 3). The robustness portion of the protocol tested for variations in phage activity (concentration), phage inoculum size, and assay incubation time (Tables 4 to 6). The assay was unaffected by these parameters within the limits tested. Sensitivity and specificity were tested by screening 51 independent B. anthracis and 49 other Bacillus species and strains (Table 7). Two sensitive non-B. anthracis strains, B. cereus ATCC 4342 and B. mycoides CDC680, along with two nonsusceptible strains of B. anthracis were included in this study (Table 1). As mentioned above, B. mycoides CDC680 was subsequently found to be a B. anthracis strain lacking both plasmids, thereby altering the original design of the study from 50 B. anthracis and 50 non-B. anthracis strains to 51 and 49 strains, respectively. Overall, the assay passed all validation acceptance criteria and was determined to be fit for its intended use and for screening of putative culture isolates to identify B. anthracis.

As stated, the choice of B. anthracis strain for use in measuring potency has been controversial due to gamma phage sensitivity variations among strains and bacterial inoculum size. As shown in Fig. 3, strains respond differently to heavy and light bacterial inocula. Those strains possessing the pXO1

### TABLE 3. Effect of phage lot on intermediate precision for identification of B. anthracis by gamma phage lysis

<table>
<thead>
<tr>
<th>Assay set (lot)</th>
<th>No. of expected positives</th>
<th>No. of observed positives</th>
<th>% Positivea</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>3</td>
<td>7</td>
</tr>
</tbody>
</table>

a Defined as positive in either the first or second quadrant.

### TABLE 4. Effect of phage concentration on robustness of identification of B. anthracis by gamma phage lysis

<table>
<thead>
<tr>
<th>Assay set (% phage)</th>
<th>No. of expected positives</th>
<th>No. of observed positives</th>
<th>% Positivea</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>5</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>90</td>
<td>5</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>80</td>
<td>5</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>70</td>
<td>5</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>60</td>
<td>5</td>
<td>1</td>
<td>5</td>
</tr>
</tbody>
</table>

a Defined as positive in either the first or second quadrant.

### TABLE 5. Effect of inoculum size on robustness of identification of B. anthracis by gamma phage lysis

<table>
<thead>
<tr>
<th>Size (load) (mm)</th>
<th>No. of expected positives</th>
<th>No. of observed positives</th>
<th>% Positivea</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>0</td>
<td>7</td>
</tr>
</tbody>
</table>

a Defined as positive in either the first or second quadrant.

### TABLE 6. Effect of incubation time on robustness of identification of B. anthracis by gamma phage lysis

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>No. of expected positives</th>
<th>No. of observed positives</th>
<th>% Positivea</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>5</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>16</td>
<td>5</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>20</td>
<td>5</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>24</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

a Defined as positive in either the first or second quadrant.
plasmid give more distinct clear plaques in the second quadrant, whereas those strains lacking the pXO1 plasmid produce distinct plaques in both quadrants. These and other observations of variation in phage susceptibility provided the basis for application of phage to both the first and second quadrants as a standard operating procedure. McCloy (9) made similar observations in which she reported that her “phage preparation had an appreciable action only if the bacterial inoculum was large,” whereas “if the inoculum was smaller, the phage produced no detectable effect after 18 h.” She goes on to state that “there were sometimes minor differences in the degree to which it (bacterial strain) was attacked; these differences might perhaps have been due the fact that the bacterial inoculum was not standardized.”

It is obvious from these studies that an accurate assessment of phage preparation activity and quality control requires the use of both pXO1-positive and pXO1-negative strains. The negative strains (such as the Pasteur strain) allow accurate plaque counts, whereas the pXO1-positive strains assess the potency of the preparation to identify fully virulent strains such as the Ames strain. The basis of the increased sensitivity of strains lacking the pXO1 plasmid compared to that of positive strains is not understood. As the pXO2 plasmid appears to have no effect on the response to the gamma phage, avirulent strains such as the Sterne strain can be used with the avirulent Pasteur strain to determine potency and to perform quality control assessments. Recent data, not reported here, suggest that the effect of the pXO1 plasmid may be more indirect. It has been our observation and that of others that strains possessing the pXO1 plasmid sporulate more readily than those lacking pXO1. As was seen in this study (data not shown), Gram-stained 18- to 20-h growth from pXO1-positive strain cultures on SBA showed high numbers of spores, whereas those from Pasteur-like strains lacking pXO1 typically showed very little, if any, spores. The net result is that growth material used from primary culture of a pXO1-positive strain to test for gamma phage sensitivity would contain larger amounts of spores, cell wall debris, and dead cells containing endospores than material from a pXO1-negative strain. In theory, when gamma phage is added to the inoculated surface, much of the phage may be binding to receptors in this debris and once bound are incapable of infecting bacilli later emerging from the germinating spores. However, when inoculated onto Pasteur-like strains, there is minimal competing debris, and phage binds to and infects viable bacilli. The net result is that fewer phages are required to generate a plaque. This is supported by our observation that when washed Sterne spores were used as the inoculum rather than growth from primary culture, clearly defined macroplaques were generated at much higher dilutions, similar to those of the Pasteur strain.

To date, gamma phage prepared using the host strain, B. anthracis CDC684, and filter sterilized using low-protein-binding 0.22-μm filters to remove bacteria has shown remarkable stability over 2 years while being refrigerated at 4°C. This is contrary to the previous findings of others (6), who found it necessary to frequently prepare fresh batches of gamma phage. The stability of gamma phage preparations produced as described here allows individual gamma phage preparations to be employed for long-term use and dispersal to multiple laboratory locations with minimal loss in activity. At this juncture, several preparations have undergone analysis for potency and pour-plate PFU per milliliter determinations. Phage lot TA101201, prepared in October 2001, showed less than a log10 drop in PFU concentration from 2001 to 2004 and retained its activity towards fully virulent B. anthracis strains when tested for potency. Likewise, other lots such as TA022303 also showed less than a log10 decrease from February 2003 to February 2004, whether measured using the potency or the pour-plate assay. Since there is less than a log10 decrease in potency, it is reasonable to assign a preparation an expiration date of at least a year if it produces distinct macroplaques when diluted 1:8 to 1:10 or more in nutrient broth and applied to the first and second quadrants of a test strain possessing the pXO1 plasmid, such as Sterne. In general, our preparations are sufficiently stable for 18 months to 2 years. According to an unpublished method dated January 1971, originally provided by W. Cherry, Analytical Bacteriology Unit, CDC, acceptable phage preparations can be used either undiluted or diluted 1:10, and phage preparations having concentrations less than 107 PFU/ml should not be used. Our observations were consistent with those statements.

The findings show the reliability of this simple biological assay for identifying B. anthracis. However, we continue to stress that confirmatory tests should be used along with this gamma phage assay for positive identification. Validation studies showed lytic behavior to be as expected for all isolates, giving an assay specificity of at least 98% for B. anthracis. Gamma phage application in the two quadrants was an essential element of robustness and tolerated variation in inoculum load. Finally, B. anthracis strain CDC684 appears to be an excellent host strain for production of batches of high-titer, stable gamma phage when produced in the manner described here.

International events have focused attention on methods for reliable and affordable methods for detecting microorganisms of biowarfare or bioterrorism concern. In 2002, the Food and

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**TABLE 7. Sensitivity and specificity of identification of B. anthracis by gamma phage lysis**

<table>
<thead>
<tr>
<th>Analysts</th>
<th>Isolate (no.)</th>
<th>No. of expected positives</th>
<th>No. of observed positives&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% Positive&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A and D</td>
<td>B. anthracis (50)</td>
<td>50</td>
<td>36/47</td>
<td>96</td>
</tr>
<tr>
<td>A and D</td>
<td>Non-B. anthracis (50)</td>
<td>0</td>
<td>2/2</td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Defined as positive in either the first or second quadrant.
<sup>b</sup> Two of 50 B. anthracis isolates were known to be nonsusceptible and were included in this study for completeness.
<sup>c</sup> Two of 50 non-B. anthracis Bacillus isolates were known to be sensitive and were included for completeness. One of the strains, Bacillus mycoides CDC680, was identified by Beverly Mangold (Tetracore, Inc.) and Paul Jackson (Lawrence Livermore National Laboratories) as a plasmid-deficient Bacillus anthracis strain.
<sup>d</sup> Values are observed results of analyst A/observed results of analyst D.

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VOL. 43, 2005 USE OF GAMMA PHAGE FOR B. ANTHRACIS IDENTIFICATION 4787
Drug Administration Division of Clinical Laboratory Devices recognized that assay methods for *B. anthracis* and *Yersinia pestis* were eligible for classification as preamendment in vitro diagnostic products. Recently, AOAC International has recognized the method using gamma phage lysis and direct fluorescence assay as satisfactory for first action as an AOAC Official Method of Analysis (Scott Coates, personal communication).

**ACKNOWLEDGMENTS**

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Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army in accordance with (IAW) AR 70-31.

**REFERENCES**
