Clostridium tetani in a Metropolitan Area: Limited Survey Incorporating a Simplified In Vitro Identification Test

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ABSTRACT

BYRD, THOMAS R. (Harvard University School of Public Health, Boston, Mass.), AND H. L. LEY, JR. Clostridium tetani in a metropolitan area: limited survey incorporating a simplified in vitro identification test. Appl. Microbiol. 14:993–997. 1966—In a limited survey, three toxigenic and one nontoxigenic strains of Clostridium tetani were isolated from 18 environmental samples from metropolitan Boston. No C. tetani was found in 100 samples of human feces, 20 samples of dog feces, and two samples of horse feces. A simple modification of the halo precipitin test was studied in conjuction with the mouse lethality test for tetanus toxigenicity and was found to be a useful, although not a wholly definitive, technique.

The literature is replete with suggestions that Clostridium tetani is virtually universally distributed in the environment, presumably as a result of contamination by the feces of domestic animals and man (10). Although Smith (15) concluded that the organism has many habitats in nature, including the soil, the idea of the horse as the prime source of the organism is still widely prevalent. Early work in this country, especially that of Bauer and Meyer (1) and Gilles (2), tended to exonerate the horse and implicate man and soil. A review of the literature revealed no substantial survey data on the distribution of tetanus spores in feces or the environment in this country since 1937.

The low incidence of clinical tetanus, the apparent low level of immunization, and the relative scarcity of sources of massive fecal contamination (such as horses) suggested that the prevalence of C. tetani in the metropolitan Boston environment might be significantly less than that predicted by the literature. Consequently, a limited study was undertaken to determine both the approximate level of contamination of the environment with toxigenic strains of C. tetani and possibly the probable source of such contamination. The study was also designed to evaluate or develop techniques adaptable to mass studies of anaerobic organisms in a field situation.

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MATERIALS AND METHODS

Isolation media. Cooked Meat Medium (Difco) was prepared according to the manufacturer’s directions and used on the day of preparation. Yeast extract-blood-agar and sorbic acid-polymixin B sulfate-thioglycolate medium (SAPB) were prepared as described by Wetzler et al. (17), except that Difco dehydrated media and defibrinated horse blood were substituted. Chloral hydrate-sodium azide inhibitory medium (CHSA) was prepared as recommended by MacLennan et al. (9), with the use of yeast extract-blood-agar as a base.

Dextrose proteose No. 3 (DP-3) Agar. A 40-ml amount of Difco Dextrose Proteose No. 3 Agar and 7 g of Difco Agar were dissolved in 1 liter of distilled water by heating to boiling and were dispensed in 8-ml amounts into tubes (16 by 150 mm). After being autoclaved for 15 min at 121 C, the media were incubated at 37 C overnight to check for sterility and then were stored at room temperature.

Tetanus antitoxin. Equine antitoxin without preservative (Massachusetts Institute of Laboratories lot No. LA 308) containing 1,660 Lf (limit of flocculation) per ml was used for halo tests. Equine antitoxin with preservative (lot No. LA 117 P, from the same source) containing 2,000 antitoxin units per ml was used for passive immunization of control mice.

Reference strains of organisms. Two reference strains of toxin-producing C. tetani were used. Reference strain S-2 is the strain used for tetanus toxoid production by the Massachusetts Institute of Laboratories and was identified as their strain D-7. Reference strain S-3 was obtained from the American Type Culture Collection, accession no. 9441. Another strain (designated S-1 by us) was received from K. F. Girard of
the Massachusetts Institute of Laboratories. Although this strain had been thought to be a toxigenic variety of C. tetani, its behavior in our hands was atypical, in that it failed to kill mice. For this reason, it must be considered a nontoxigenic strain, at least in the media which were employed in this study.

Mice. Strain CD-1 female white mice, weighing 18 to 20 g (obtained from Charles River Mouse Farms), were used to test for toxigenicity.

Sources of specimens. The sources of fecal and environmental specimens are described in detail in the Results section.

Isolation techniques. Isolation methods were essentially those of Wetzel et al. (17), modified in the case of environmental specimens by the initial growth period recommended by Gilles (3). Incubation temperatures for isolation media were 37°C throughout.

Approximately 1 g of each fecal specimen was inoculated into 8 ml of Cooked Meat Medium, covered with 2 cm of sterile mineral oil, and incubated for 24 to 48 hr. Approximately 20 g of each environmental specimen was suspended in 40 to 45 ml of sterile saline, heated at 80 to 82°C for 1 hr, and added to 250-ml flasks containing 95 ml of Cooked Meat Medium. Environmental cultures were covered with sterile mineral oil to a depth of 2 cm and were incubated at 37°C for 10 days before subculturing.

Subcultures were made by pipetting approximately 0.5 ml of the master culture into SAPB. After aerobic incubation for at least 24 hr, 4-mm loopfuls of the SAPB cultures were streaked onto CHSA plates. The plates were incubated for 48 hr in a Brewer anaerobic jar. Representative colonies of all morphological types were inoculated into thioglycolate broth and incubated for 24 hr. Gram stains were made, and all broth cultures of gram-positive bacilli were streaked onto yeast extract-blood agar plates which were incubated aerobically to ascertain the sensitivity of the organisms to oxygen. Any broth culture containing anaerobic, gram-positive bacilli was streaked onto another yeast extract-blood-agar plate and incubated anaerobically so that colonial characteristics and purity of culture could be observed. Subcultures were made into Cooked Meat Medium, covered with sterile mineral oil, and incubated 10 days for toxigenicity studies.

Techniques for identification of toxigenic C. tetani. Identification was made by means of the mouse lethality test and the "halo" precipitin test.

For the mouse lethality test, the fluid portion of 10-day cooked-meat cultures of test organisms was mixed with an equal quantity of sterile 2.5% aqueous calcium chloride solution. A 0.3-ml amount of this mixture was injected into the muscles of the left thigh of each of two immunized and two unimmunized mice. Passively immunized mice were injected with 500 units of tetanus antitoxin administered intraperitoneally 1 to 4 hr before injection of presumed C. tetani. Mice were observed frequently for signs of tetanus for a period of 7 days. Each mouse test included reference strains as controls.

For the halo precipitin test, 8-ml tubes of DP-3 Agar were melted, cooled, and inoculated with 2-mm loopfuls of 3- to 5-day thioglycolate broth cultures of organisms obtained in the manner described above. While still liquid, these cultures were poured into sterile petri dishes (15 by 100 mm) into which had been placed 4 drops of tetanus antitoxin without preservative (final concentration of antitoxin in the medium in the plate was approximately 40 Lf/ml). The plates were swirled gently to mix the antitoxin with the media, allowed to solidify, and incubated at 37°C in Brewer jars. The plates were examined at intervals from the 3rd through the 7th day of incubation, by use of a dissecting microscope, indirect lighting, and a black background.

RESULTS

Samples of feces were collected from 20 dogs at a veterinary hospital in the Boston area, representing dogs admitted for grooming as well as for various treatment procedures. Thirty-five aerobic strains and 17 gram-positive anaerobic bacilli were recovered from these fecal samples. None of the anaerobes could be identified as toxigenic C. tetani by mouse or halo test.

Four anaerobic isolates from two samples of horse feces obtained from the stables at the Massachusetts Institute of Laboratories failed to yield organisms identifiable as C. tetani by mouse or halo test.

A summary of the isolations of anaerobes from environmental samples is presented in Table 1. Two areas were selected for sampling. Area A (the vicinity of the Massachusetts Institute of Laboratories, which maintains a number of

<table>
<thead>
<tr>
<th>Environment</th>
<th>Samples</th>
<th>Anaerobes isolated</th>
<th>Clostridium tetani isolated</th>
<th>Strain designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Soil</td>
<td>5</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>Sweeping</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Soil</td>
<td>6</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Sweeping</td>
<td>4</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>18</td>
<td>22</td>
<td>4</td>
</tr>
</tbody>
</table>

* Area A: an area of probable high contamination by horse feces. Area B: an area not subjected to direct contamination by horse feces in the recent past.

b Strain 4A was obtained from a fenced area used to exercise horses and 9A from an open driveway ca. 90 meters distant. Strain 11A was obtained from a cultivated flower bed and 14B from an open area. The sites of isolation of the last two strains were each approximately 45 meters from a small sluggish stream.

c Sweepings were obtained by brushing roadway areas to collect approximately 20 g of dust and dirt.
horses) was chosen as an environment obviously or potentially contaminated with horse feces. Area B (the vicinity of Park Drive and the Fenway, Boston) was chosen as an area not obviously directly contaminated by horse feces in the recent past, but subject to fecal contamination by small domestic pets, squirrels, and birds. Three strains of *C. tetani* which yielded positive results with both the halo test and mouse inoculation were recovered from these environmental samples, as well as a fourth strain which did not kill mice but gave a positive halo reaction.

Table 2 summarizes the results obtained when 43 wild strains of gram-positive anaerobic bacilli and three reference strains of *C. tetani* were subjected to both the halo and mouse lethality tests.

A comparison of the behavior of our stock and wild strains of *C. tetani* is presented in Table 3. The atypical strain, S-1, consistently grew as a thin film on the surface of the agar, without producing discrete colonies. Strain S-2 did not swarm. Strain S-3, as well as the wild strains, produced characteristic colonies as well as swarming.

The halos noted in Table 3 began to appear at about 3 days of incubation and continued to intensify with time. By the 7th day of incubation, they were in all cases readily identifiable by the naked eye with the use of transillumination. Removal of the plates to an aerobic environment after 5 to 7 days of anaerobiosis served to intensify the halos further. Reactions were easily identifiable for at least 3 weeks if the plates were kept at room temperature and protected from contamination.

Figure 1 illustrates the precipitin halos produced by stock strain S-2 of *C. tetani*. Because of the swarming tendency of *C. tetani*, it was necessary to use media containing 2% agar to inhibit swarming sufficiently to allow the production of halos. Several readily available commercial media were evaluated, and DP-3 with 2% agar was found to be most satisfactory. The other media tested included Nutrient Agar, PPLO Agar, and Liver Veal Agar (all Difco products).

The results of mouse lethality tests are also summarized in Table 3. Toxigenic reference strains S-2 and S-3 killed unimmunized mice and produced typical symptoms of tetanus in these animals. Mice protected with antitoxin were not

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### Table 2. Comparison of 43 wild strains of gram-positive anaerobic bacilli and three reference strains of Clostridium tetani by halo and mouse lethality tests

<table>
<thead>
<tr>
<th>Halo test</th>
<th>Mouse lethality test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. positive</td>
</tr>
<tr>
<td>Wild strains</td>
<td></td>
</tr>
<tr>
<td>No. positive</td>
<td>3 (4A, 9A, 11A)</td>
</tr>
<tr>
<td>No. negative</td>
<td>0</td>
</tr>
<tr>
<td>Reference strains</td>
<td></td>
</tr>
<tr>
<td>No. positive</td>
<td>2 (S-2, S-3)</td>
</tr>
<tr>
<td>No. negative</td>
<td>0</td>
</tr>
</tbody>
</table>

* Strain designations are shown in parentheses.

### Table 3. Comparison of three reference and four wild strains of Clostridium tetani

<table>
<thead>
<tr>
<th>Strain</th>
<th>Swarming on</th>
<th>Halo test</th>
<th>Mouse lethalitya</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CHSAa</td>
<td>BAb</td>
<td>Immunized</td>
</tr>
<tr>
<td>S-2</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>S-3</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4A</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9A</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>11A</td>
<td>−</td>
<td>+</td>
<td>0/2 2/2</td>
</tr>
<tr>
<td>14B</td>
<td>++</td>
<td>++</td>
<td>0/2 1/2</td>
</tr>
<tr>
<td>S-1</td>
<td>++</td>
<td>++</td>
<td>0/4 0/4</td>
</tr>
</tbody>
</table>

a Chloral hydrate-sodium azide-blood-agar.

b Yeast extract-blood-agar.

Number of mice dying/number of mice inoculated. All mice died with symptoms of tetanus except the single animal which died after injection of strain 14B.
killed by these strains, except in the first test performed with strain S-3 in which one of four mice died. In that single test, the protective antitoxin was administered immediately before inoculation of the organisms. Our interpretation of the aberrant death of this single animal is that it resulted from an overwhelming dose of preformed toxin, which killed the animal before protective amounts of antitoxin could be absorbed from the peritoneal cavity. In all subsequent tests in which antitoxin was administered 1 to 4 hr before injection of the organism, all mice receiving antitoxin survived.

Three of the wild strains of C. tetani (4A, 9A, and 11A) also produced results comparable to those observed with reference toxigenic strains S-2 and S-3. The fourth wild strain (14B) killed one of two unprotected mice in one test. The death, which occurred on the 4th day after inoculation, is believed to be nonspecific, because the animal demonstrated no symptoms of tetanus. Wild strain 14B, therefore, is believed to be similar to the nontoxigenic reference strain S-1 in that it forms halos without producing toxin.

One hundred samples of human feces were obtained from the Diagnostic Laboratories at the Massachusetts Institute of Laboratories. These were specimens submitted in buffered glycerol-saline for routine examination for enteric bacteria, and were inoculated into Cooked Meat Medium at from 2 to 10 days after the original collection. Specimens were equally divided between males and females. The age range of the patients was from 1.5 months to 82 years, with a mean age of 13.5 years. Ninety aerobic strains and 14 gram-positive anaerobic bacilli were recovered from these feces. Of the anaerobes, none gave positive halo reactions. Mouse toxicity tests were not performed on these organisms because of the results reported above, which indicate that no positive mouse tests may be expected with organisms not producing halos.

**DISCUSSION**

The mouse lethality test used in these experiments was not as refined as some described (14), in that the inoculum was presumably a mixture of preformed toxin, vegetative organisms, and spores. However, it gave very dependable results with control inocula, provided that inoculation was made 1 hr or more after passive immunization of the mice.

The halo phenomenon, described by Petrie and Steabben (11, 12), has been in use at the Massachusetts Institute of Laboratories for several years. The modification described here was developed as a result of the need for a simple identification test, not requiring animals, complex media, or large amounts of equipment and expenditures of time. It appears to be reasonably specific, and is particularly useful for obtaining pure cultures of toxigenic C. tetani from mixtures containing closely related organisms. In view of its simplicity, it should prove useful in field or clinical laboratories for the presumptive identification of C. tetani.

Lowbury and Lilly (8) have proposed and used another technique for identification of C. tetani, which they term an “antitoxin-controlled blood agar plate.” In their procedure, a pure culture of an organism suspected of being C. tetani is streaked on a blood-agar plate divided into halves, one of which contains antitoxin. Strains of C. tetani produce zones of hemolysis in the blood-agar in the absence of antitoxin, and hemolysis is inhibited on the half of the plate containing antitoxin. In contrast to the halo technique, the hemolysis-inhibition test is of limited value in separating mixed cultures of anaerobes. Furthermore, recent work of Hardegree (4) has demonstrated that the hemolysin produced by C. tetani can be separated by gel filtration from the neurotoxin produced by the organism. For this reason, a positive result with the hemolysin-inhibition test does not necessarily indicate a toxin-producing organism unless the production of both toxin and hemolysin are invariant characteristics of all toxigenic strains. In our hands, with horse blood-agar media, production of hemolysis was an erratic phenomenon among both the reference and the wild strains of C. tetani studied. We therefore prefer the mouse toxicity test as the definitive procedure for identification of toxigenic strains of C. tetani.

We had hoped that the halo test described would prove to be as dependable as the mouse lethality test for identification of toxigenic C. tetani. However, the results observed with both reference strain S-1 and wild strain 14B clearly demonstrate that the halo test will occasionally give positive reactions with strains which will not kill mice. The reason for these “false-positive” halo reactions is not apparent at this time. However, it is of interest that Latham et al. (7), in their studies of gel filtration of tetanus toxoids, have demonstrated the presence of small amounts of a material in tetanus toxoids which is capable of reacting with commercial tetanus antitoxins in both tube and double agar-diffusion tests to form precipitates, but which is poorly antigenic in protecting animals against lethal challenge with tetanus neurotoxin. This material, which Latham called Fraction III, appears in gel filtration eluates at approximately the position (in respect to the protective and highly antigenic specific tetanus toxoid) comparable to Hardegree’s hemolysin.
We therefore hypothesize that our strains S-1 and 14B produce halos because they produce hemoly-
sin but do not kill mice because they do not produce toxin. We are not in a position to test
this hypothesis.

No valid conclusions can be drawn from the failure to find C. tetani in fecal samples, although the
results were not altogether unexpected. Some investigators have reported finding C. tetani in
significant proportions of human fecal specimens (1, 16), but most later studies have yielded nega-
tive results (5, 6, 13). An exception to this general-
ization is the report of Lowbury and Lilly (8), who
isolated C. tetani from 10 of 35 specimens of hu-
man feces obtained in Birmingham, England. Pos-
sible reasons for our negative results in examina-
tion of human fecal specimens have been con-
sidered. If tetanus organisms were present in
vegetative forms in the fecal specimens, they
could have been killed during the long period of
aerobiosis between collection and culturing. The
extensive overgrowth by enterococci, which ap-
peared to be essentially uninhibited by the tech-
niques used, could have obscured tetanus organ-
isms present in small numbers.

The frequency of isolation of C. tetani from the
environment is approximately that which would
be expected on the basis of similar studies re-
ported by Gilles (2). Although the possibility of
contamination of area B through the use of
horse manure as fertilizer cannot be excluded,
the similarity of the findings in the two areas sup-
ports the thesis that perhaps the horse is not an
important factor in the distribution of C. tetani in
metropolitan Boston.

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