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Novel Method for Measuring Growth of *Treponema hyodysenteriae* and Its Application for Monitoring Susceptibility of Clinical Isolates to Antimicrobial Agents

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A microdilution assay method was developed to determine MICs of antibiotics for *Treponema hyodysenteriae* and to estimate the bacterial burden of mice colonized by this swine pathogen. Hemolysis of bovine erythrocytes in broth was correlated with growth of the spirochete, and growth inhibition by drugs could be determined by measuring hemolysis. MICs of 10 antibiotics for eight clinical isolates of *T. hyodysenteriae* measured by this method were similar to values obtained by an agar dilution method and to values reported in the literature. This method was also used to demonstrate the elimination of *T. hyodysenteriae* from the ceca of infected mice following oral treatment with carbadox. The procedure described in this paper provides a rapid and simple method for determining MICs of antibiotics for *T. hyodysenteriae* and may be adapted for the purpose of determining the presence of this veterinary pathogen in cecal contents of experimentally infected mice.

Treponema hyodysenteriae is the causative agent of swine dysentery, a mucohemorrhagic enteric disease of economic importance in the swine industry (1). Originally classified in the genus *Treponema*, reclassification of this organism into a new genus has recently been suggested on the basis of new genetic and molecular biology data (9). Although swine dysentery is controlled in part through the use of antibiotics, the susceptibilities of clinical isolates to drugs are not routinely determined in most laboratories, perhaps because of the reputation of this organism as a fastidious anaerobe. Studies reported to date (4, 5) have used the agar dilution technique to determine the MICs of antibiotics for *T. hyodysenteriae*. Broth dilution techniques using a microdilution format are less labor and resource intensive and are compatible with automated systems for testing large numbers of isolates. Such a method would be useful in obtaining information on the susceptibilities of clinical isolates of *T. hyodysenteriae* to antibiotics. This information may be of use for monitoring emerging resistance trends and may aid in the selection of appropriate drugs for therapy and/or prevention in infected herds. Recent advances in knowledge of the physiology of *T. hyodysenteriae* have permitted the development of simple and reliable methods for its cultivation in broth but have shown that growth in broth under strict anaerobic conditions is erratic (8, 10). This unique organism requires small amounts of oxygen for optimal growth (10). Thus, MICs determined by broth dilution under anaerobic conditions may be difficult to reproduce. *T. hyodysenteriae* is known to elaborate a hemolysin which produces beta-hemolysis when the organism is grown on blood-containing media. This hemolysin has been characterized by a number of investigators (3, 6, 7). In this report, we show that the hemolysis of bovine erythrocytes in a liquid medium is correlated with anaerobic growth of *T. hyodysenteriae* and that this correlation can be exploited by using a microdilution format to determine MICs of antibiotics and to monitor the bacterial burden in the ceca of mice colonized by this organism.

MATERIALS AND METHODS

Spirochetes were grown in brain heart infusion broth (BBL) plus 10% (vol/vol) fetal bovine serum (BHIS), prepared anaerobically under 10% CO_2–90% N_2. The medium was dispensed into septum-stoppered glass tubes (120 by 16 mm), and approximately 1% O_2 was added to the headspace by injecting an appropriate volume of sterile room air. Each tube contained 5 ml of medium. Brain heart infusion broth plus 2.5% serum and 2.5% bovine erythrocytes (washed three times in phosphate-buffered saline [PBS], pH 7.4) (BHIS-RBC) was used to monitor growth by hemolysis (see below).

Inoculum for in vitro studies was prepared by growing *T. hyodysenteriae* strains overnight in BHIS at 37°C with continuous mixing at a moderate rate by means of a magnetic stirrer. Cultures were diluted to an optical density (OD) at 660 nm of 0.2 and then diluted 1:100 with PBS. Final cell density was approximately 10^6 CFU/ml, as determined by viable counts. This procedure was used to prepare inocula for both agar and broth dilution methods. Growth studies were conducted in 96-well microtiter trays containing 150 μl of BHIS-RBC in each well. A 25-μl aliquot of cell suspension was used to inoculate wells in microtiter trays which were then transferred to an anaerobic chamber (Coy Lab Products) and incubated in plastic bags at 37°C for 72 h. The turbidity of each well was determined by measuring the A_{600} with a Biotek EL310 plate reader. Hemolysis was measured by subtracting the absorbance of inoculated wells from the absorbance of uninoculated wells (ΔOD). For viable counts, 100-μl aliquots were removed from three wells, diluted, and plated on BHIS agar containing 5% (vol/vol) washed bovine erythrocytes. Colonies were counted after incubation in an anaerobic chamber for 4 days at 37°C. For growth studies, a series of microtiter trays was prepared, and at several different times from 0 to 72 h, one plate was removed,

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hemolysis was measured, and wells were sampled at random for viable counts. To examine the correlation between growth inhibition and inhibition of hemolysis, sub-MIC levels of carbadox or penicillin G were used to achieve various levels of growth inhibition. Growth was then measured by determining the degree of hemolysis as described above and by determining the number of spirochetes present by means of direct counts. Direct counts were conducted by using a Petroff-Hausser counting chamber.

For determination of the MICs of antibiotics, serial twofold dilutions of drugs in BHIS-RBC were carried out in 96-well microtiter trays. These trays were incubated and read spectrophotometrically in the same manner as plates used for growth studies. To test for hemolysis resulting from direct action of drugs on erythrocytes, uninoculated control wells containing the highest concentrations of drugs were included. Additional control wells were inoculated but received no drug. To determine MICs by the agar dilution method, serial twofold dilutions of drugs were prepared in sterile distilled water and 1 ml of each dilution was added to duplicate sterile petri dishes along with 9 ml of BHIS-RBC supplemented with 1% (wt/vol) agar. The plates were dried overnight in the dark at room temperature. After inoculation with *T. hyodysenteriae* isolates by means of a multiple-inoculation device (Cathra Systems, Inc.), plates were transferred to the anaerobic chamber and incubated for 72 h at 37°C. The lowest concentration of an antibiotic which inhibited hemolysis in either broth or agar was considered the MIC. The MICs of 10 selected antibiotics were measured for eight clinical isolates by both the agar and broth dilution techniques. With the exception of penicillin G, novobiocin, and chloramphenicol, antibiotics were chosen for testing on the basis of their use for the control and/or prevention of swine dysentery (1).

The procedure used for in vivo studies was similar to that reported by other investigators (2, 11). Briefly, female NSA mice weighing approximately 18 g (Harlan Sprague Dawley, Indianapolis, Ind.) were challenged orally with 1.0 ml of an overnight broth culture of *T. hyodysenteriae* containing approximately 5×10^8 viable cells per ml. Culture was administered twice on the day of challenge, once in the morning and once in the afternoon. Carbadox was also administered twice daily by the oral route, beginning 3 days after challenge and continuing for 4 consecutive days. Nonmedicated animals received saline. On the day after the final drug dose, mice were euthanized by CO_2 asphyxiation, and the ceca were removed, diced with sterile scissors, and placed in tubes containing glass beads and BHI. After vigorous vortexing of the cecal samples, 75 μl of homogenate was added to 150 μl of BHIS-RBC containing rifampin (25 μg/ml) and spectinomycin (400 μg/ml) in a 96-well microtiter tray. Serial threefold dilutions were then carried out for each sample, and the trays were incubated in an anaerobic chamber for 72 h at 37°C. The presence of *T. hyodysenteriae* in the samples was determined by the presence of hemolysis in wells beyond the second dilution. To show the validity of the hemolysis assay for detecting colonization of mice by *T. hyodysenteriae*, ceca from noninfected mice were homogenized and supplemented with pure cultures of *T. hyodysenteriae*. These spiked samples were then assayed for the presence of spirochetes by the procedure described above. The presence of spirochetes in hemolyzed wells was confirmed by microscopic observation. Untreated cecal contents were examined in the same manner.

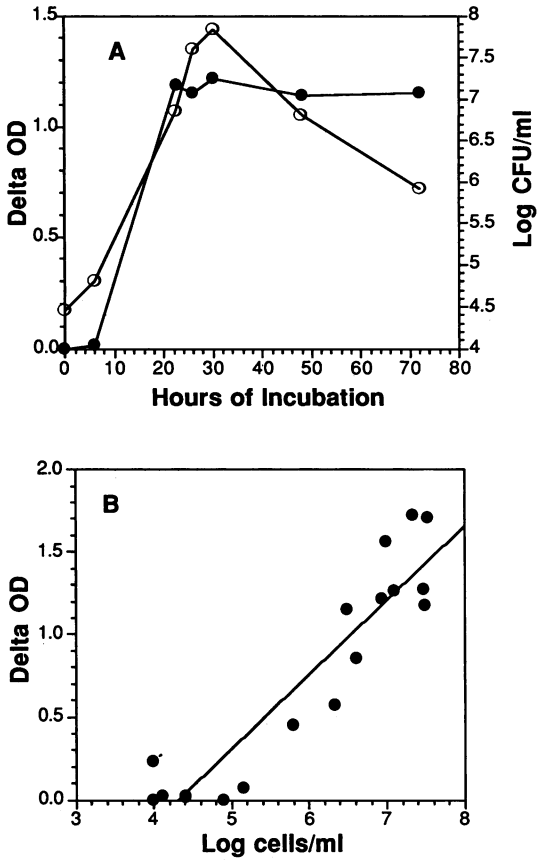

FIG. 1. Relationship between hemolysis and growth of *T. hyodysenteriae*. (A) Growth measured by hemolysis (ΔOD [●]) and cell number by viable count (log CFU per milliliter [○]). Each point is the average of two experiments. (B) Correlation between hemolysis (ΔOD) and cell number by direct count (log cells per milliliter) after incubation for 24 h in the presence of sub-MIC levels of penicillin G or carbadox. Data for both drugs and duplicate experiments were pooled. Correlation coefficient = 0.929; $P = 0.0001$.

RESULTS AND DISCUSSION

Growth of *T. hyodysenteriae* in liquid medium containing bovine erythrocytes was monitored by measuring the decrease in absorbance which occurred as a result of hemolysis. The decrease in absorbance due to hemolysis was accompanied by an increase in cell number, and the magnitude of the change in OD was correlated with the number of spirochetes present (Fig. 1). Growth of *T. hyodysenteriae* in BHIS under anaerobic conditions has been reported to be erratic unless approximately 1% oxygen is included in the headspace gas (10). In our studies, growth in BHIS-RBC was reproducible and consistent in the absence of added oxygen. Although a hydrogen-oxygen monitor in our anaerobic chamber read 0 ppm oxygen at all times during growth studies, it is likely that small amounts of oxygen were initially present in the medium, since microtiter trays were inoculated on the bench top under standard atmospheric conditions. Other than incubation of the microtiter trays under anaerobic conditions, no special precautions or medium-handling procedures were needed to ensure reproducible growth. Spirochetes grew equally well when microtiter trays were incubated in Gas-Pak jars and in a Coy anaerobic

TABLE 1. IC_{50}s and IC_{90}s of carbadox and penicillin for *T. hyodysenteriae* B141TP[a]

Antibiotic (MIC [μg/ml])	Method of growth measurement	IC (μg/ml)	
		50%	90%
Carbadox (0.05)	Hemolysis	0.01	0.04
	Direct count	0.02	0.05
Penicillin (0.78)	Hemolysis	0.32	0.71
	Direct count	0.41	1.00

[a] ICs calculated from hemolysis (ΔOD) or direct count (cells per milliliter) data by a regression technique.

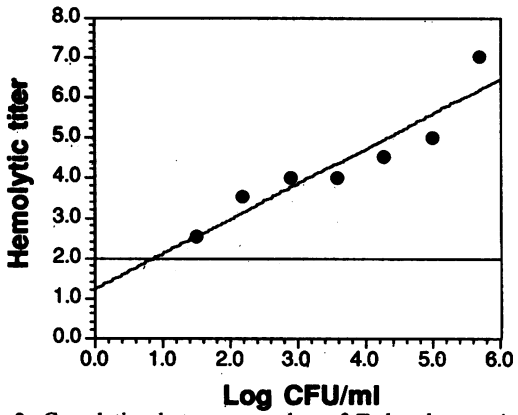

FIG. 2. Correlation between number of *T. hyodysenteriae* cells added to mouse cecal homogenates and the hemolytic titer, expressed as the number of dilutions which were positive for hemolytic activity. Correlation coefficient = 0.944; $P = 0.0004$. The horizontal line represents the hemolysis in cecal samples in the absence of added spirochetes.

chamber (data not shown). Thus, in spite of the unusual growth requirements of *T. hyodysenteriae*, this procedure is rapid and simple and does not require expensive or unusual equipment for anaerobiosis.

Both hemolysis (measured by ΔOD) and cell number (measured by direct count) responded in a dose-dependent fashion to sub-MIC levels of carbadox and penicillin (Fig. 1). Concentrations of antibiotic which inhibited hemolysis by 50% (IC_{50}s) and IC_{90}s calculated from ΔOD and cell number data by means of a regression analysis procedure were similar (Table 1). Thus, inhibition of growth by antibiotics resulted in inhibition of hemolysis. Note the similarity between the MICs and IC_{90}s for carbadox (0.05 and 0.04, respectively) and penicillin (0.78 and 0.71, respectively).

MICs of antibiotics determined by inhibition of hemolysis in the broth dilution procedure were similar to values obtained by the agar dilution method (Table 2). Data obtained in this study by both methods were similar to published values (4, 5). The agar dilution procedure also relied on hemolysis to monitor growth, since colonies of *T. hyodysenteriae* were essentially invisible on solid media except for the zone of hemolysis they produced; thus, it is not surprising that the two methods produced similar results.

There was a significant correlation between the number of spirochetes added to cecal samples from uninfected mice and the hemolytic titer expressed as the number of dilutions which demonstrated hemolysis (Fig. 2). Although there was some hemolytic activity in untreated cecal samples, this activity was consistently low, with a hemolytic titer of 2 or less. Spirochetes were observed only rarely in untreated cecal samples and were easily distinguished from *T. hyodysenteriae* by morphological characteristics. Endogenous spirochetes were smaller and had a greater degree of cell coiling than did *T. hyodysenteriae* spirochetes. Thus, it was apparent that this technique could be used to detect *T. hyodysenteriae* in the ceca of infected mice. Spirochetes were routinely detected in cecal homogenates from infected mice, and treatment of infected mice with carbadox at 2.5 mg or more per kg of body weight completely eliminated *T. hyodysenteriae* from the ceca (Table 3), which is consistent with the potent antibacterial activity of this compound.

An assay for monitoring the growth of *T. hyodysenteriae* has been described, and its usefulness for determining the susceptibility of clinical isolates to antibiotics used to control swine dysentery has been demonstrated. Since this is a liquid-based procedure, it can be adapted for use with automated liquid-handling systems, making testing of the antimicrobial susceptibilities of large numbers of clinical isolates practicable. This will allow monitoring of resistance development and will aid in the appropriate selection of therapeutic agents. The hemolysis assay is also a rapid and useful method for determining the colonization status of infected mice, which generally do not develop clinical symptoms of *T. hyodysenteriae* infection. With appropriate validation, the assay could be used to identify disease vectors such as wild rodents or insects and thus aid in epidemiological studies. It may be possible to adapt this method for use with swine as a diagnostic tool to identify carrier animals.

TABLE 2. Range of MICs of selected antibiotics against eight clinical isolates of *T. hyodysenteriae* determined by agar dilution and microtiter broth dilution methods

Antibiotic	MIC (μg/ml) obtained by dilution method	
	Agar	Broth
Metronidazole	<0.20	<0.20–0.39
Furazolidone	<0.20	<0.20
Carbadox	<0.01–0.03	<0.01–0.05
Tiamulin	0.05–0.10	0.03–0.10
Tylosin	25–>100	3.13–>100
Penicillin G	0.39–1.56	<0.20–0.39
Novobiocin	50–200	50–100
Gentamicin	3.13–25	3.13–12.5
Lincomycin	0.78–100	0.39–>100
Chloramphenicol	0.78–1.56	1.56

TABLE 3. Colonization of mice by *T. hyodysenteriae* after treatment with carbadox

Antibiotic treatment	Dose (mg/kg/day)	No. of culture-positive mice ($n = 10$)	Average hemolytic titer
None		7	8.4
Carbadox	0.6	8	8.9
	1.25	4	8.3
	2.5	0	<2
	5.0	0	<2

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