Rapid Microbiological Assay for Chloramphenicol and Tetracyclines

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An agar diffusion assay for chloramphenicol and tetracyclines is described, using inhibition of hemolysis by *Clostridium perfringens* as a biological indicator of antimicrobial activity. Well-defined zones formed after a 1.5- to 2-h incubation in an anaerobic chamber or after 3 h using a GasPak system. Serum levels of chloramphenicol as low as 2.0 µg/ml could be reproducibly determined with this method. Concurrent presence of gentamicin did not influence the assay. Levels of penicillin G were also reproducibly determined with this method, although use of stable zones of growth inhibition which would form after overnight incubation were a more convenient end point for this bactericidal drug. The hemolytic assay with *C. perfringens* is a reproducible, accurate, and rapid method for determination of levels for chloramphenicol and tetracyclines.

A recrudescence of interest in the therapeutic use of chloramphenicol and the parenteral tetracyclines has been awakened by the development of ampicillin resistance in *Haemophilus influenzae* (2, 18) and the recognition of the importance of *Bacteroides fragilis* in intraabdominal sepsis. Accurate determination of serum chloramphenicol levels would be advantageous for the following reasons. (i) Bone marrow suppression appears to be more likely with serum concentrations greater than 15 to 20 µg/ml (3, 15). (ii) The minimum inhibitory concentration for *B. fragilis* may range from 4 to 12 µg/ml (11). This narrow toxic/therapeutic ratio would make individualization of chloramphenicol dosage desirable in patients with anaerobic infections. (iii) Finally, measurement of serum levels would be beneficial in patients with hematologic disorders and in those with impaired clearance mechanisms due to hepatic disease, renal insufficiency, or in neonates who are prone to develop the Gray syndrome (19).

In the past, serum chloramphenicol levels have been determined by tube dilution (8), turbidimetric (9, 20), and by agar diffusion techniques (12, 16). These microbiological assays have not attained widespread usage because of their technical complexity (9, 20), low sensitivity (12), prolonged incubation times, and interference by concurrently present antimicrobial agents. The colorimetric (1, 6) and gas-liquid chromatographic methods (13) require more technical sophistication than is generally available in a hospital laboratory. This report describes a rapid accurate agar diffusion assay for

chloramphenicol and tetracyclines using *Clostridium perfringens* as the test organism.

MATERIALS AND METHODS

Assay organism. A strain of *Clostridium perfringens* (SAL no. 249) with an minimum inhibitory concentration to chloramphenicol of 0.5 µg/ml was selected as the assay organism. This strain was isolated from a clinical specimen by anaerobic chamber techniques and identified according to the criteria of the VPI Anaerobic Laboratory Manual (7). The organism was tested by a previously published method (17) for susceptibility to penicillin G, chloramphenicol, tetracycline, and gentamicin (minimum inhibitory concentrations, 0.2, 0.5, 0.06, and >32 µg/ml, respectively).

A stock culture was maintained on a prereduced anaerobically sterilized brain heart infusion agar slant (Scott Laboratories, Fiskeville, R.I.). The seed culture was prepared by transferring six to seven colonies from a brain heart infusion agar slant subcultured from the stock brain heart infusion slant 24 h previously into 5 ml of prereduced brain heart infusion broth under CO₂, followed by incubation at 37 C for 6 h. Thioglycolate broth (BBL, Cockeysville, Md.) could be used as an alternative medium if a CO₂ inoculator is not available.

Assay medium. A modified Trypticase soy agar was prepared by adding 26.66 g of Trypticase soy agar base (BBL) to 1,000 ml of distilled water and adjusting the pH to 7.3. A volume of 18.4 ml of molten agar was poured into 25-ml glass tubes and autoclaved. Following cooling to 50 C, 1 ml of whole defibrinated sheep blood (5% vol/vol) and 0.6 ml of seed culture were added, mixed, and poured onto plastic petri dishes (150 by 15 mm). The medium was allowed to harden on a level bench, and 4-mm diameter wells (maximum of 25/plate) were aspirated

with a well cutter. Additional assay media were made by adding 64 μg of gentamicin per ml (Schering Corp., Bloomfield, N.J.).

The prepared plates can be sealed in plastic bags and stored at 4 C in an aerobic environment for up to 4 days. Further storage leads to deterioration of the plates with poor microbial growth. Shelf life may be prolonged to 7 to 10 days with storage under anaerobic conditions in a large GasPak jar. With the limited shelf life, plates should be prepared twice weekly.

Assay procedure. Standard concentrations of chloramphenicol (Parke, Davis & Co., Detroit, Mich.) were added to human serum in concentrations from 64 to 0.5 $\mu\text{g}/\text{ml}$. Other diluents corresponding to the unknown, such as urine, cerebrospinal fluid, pleural or peritoneal fluids, may be used. Tetracycline and penicillin G standards were similarly made in concentrations from 8.0 to 0.06 $\mu\text{g}/\text{ml}$ and from 128 to 1 $\mu\text{g}/\text{ml}$, respectively. Wells with volumes of 35 to 40 μl were filled by capillary action from micropipettes. Standards were prepared in duplicate and test samples in triplicate per plate. Normal serum was used as the control. To determine if the simultaneous presence of gentamicin would influence the assay results, each drug was also tested with media containing this agent. Duplicate plates were made for each assay for incubation by two methods. The plates were incubated in a GasPak 150 anaerobic system (BBL) containing two GasPaks and three baskets of palladium catalyst at 37 C for 3 h. Duplicate plates were incubated at 37 C for 1.5 to 2 h inside a heated anaerobic glove box (Coy Manufacturing Co., Ann Arbor, Mich.). Zone sizes were read with a Bausch and Lomb measuring magnifier, and the diameters of the zone sizes for each concentration were averaged and plotted on semilogarithmic graph paper.

Comparison of the method with a standard microbiological assay. To test the validity of hemolysis as an indicator of antimicrobial activity in agar diffusion assays, serum samples containing penicillin G were concurrently assayed comparing results of this rapid method with two other methods: (i) a 24-h assay performed by reincubation of the rapid assay plates for an additional 22 h, at which time zones of inhibition of macroscopic colonial growth would form; and (ii) a standard assay with *Bacillus globigii* as the test organism (21).

Precision of the assay. The precision of the assay was assessed by two methods: (i) microbiologically by correlation of prepared coded concentrations with the assayed concentrations and (ii) by comparing the results of the microbiological assay with those of the colorimetric assay of Bessman (1) using paired serum samples. The linear least-squares regression lines were determined by a Hewlett Packard 9830-A computer.

RESULTS

Assay for chloramphenicol. Hemolysis was detected after 3 to 3.5 h of incubation in the GasPak jar and after 1.5 to 2 h in the anaerobic glove box. The translucent agar was converted

to a transparent one, except for a clearly defined zone of inhibition of hemolysis around the inoculated wells (Fig. 1). Prolonged incubation (8 to 24 h) produced a diminution of zone size; a similar but much less pronounced effect was observed if the plates were left on a bench at room temperature. On incubation for 18 to 36 h, macroscopic growth was noted with indistinct end points around the wells. Therefore, for optimal results, the zones were read after 3 to 3.5 h of incubation in GasPak jars or 1 to 1.5 to 2 h in the glove box. Refrigeration of the plates at 4 C after incubation preserved the intact erythrocytes so that the early results were still apparent after 24 to 48 h.

Zone size variation among replicate samples was less than 0.5 mm, with zone sizes ranging from 6 to 30 mm (Fig. 1). The mean diameters of the zones of inhibition of hemolysis plotted against the standard concentrations on triple cycle semilogarithmic graph paper produced a straight line from concentrations of 2 to 64 $\mu\text{g}/\text{ml}$ (Fig. 2). Control normal sera containing no antibiotic produced a thin rim of inhibition of hemolysis around the wells, which was consistent from sample to sample. Control sera from patients with diabetes, renal insufficiency, and collagen vascular diseases did not produce wider zones of inhibition. The diameter of the control sera zone was less than 6 mm in diameter and did not affect the standard curve. Cerebrospinal fluid, urine, and phosphate-buffered saline did not produce this zone, suggesting

FIG. 1. Zones of inhibition of hemolysis formed after a 90-min incubation in the anaerobic glove box. The translucent agar became transparent, except for the zones around the inoculated wells.

FIG. 2. Regression curve of chloramphenicol concentration versus zone size diameter showing the linear relation from 2 to 64 $\mu\text{g/ml}$.

that the principle(s) inhibiting hemolysis resided in the serum protein fraction. The lowest readable concentration was 1.0 $\mu\text{g/ml}$, although the most consistent results were from concentrations equal to or greater than 2.0 $\mu\text{g/ml}$.

Preincubation of the plates for 30 min to allow for diffusion did not yield significantly different results from that of immediate incubation. Freshly poured plates produced the most rapid results; refrigeration of the plates for up to 4 days prior to use gave consistent results, although the incubation time had to be increased to 4 to 6 h.

Precision of the assay. The precision of the assay was assessed microbiologically by correlation of the prepared concentrations with assayed concentrations on the basis of 39 samples prepared to contain from 1 to 100 μg of chloramphenicol per ml. The correlation of the prepared concentration with the assayed concentration was 0.997 ($P < 0.001$). The slope of the linear regression line was 1.01, not significantly different from 1.00 ($P > 0.95$). The Y intercept (0.04) was not significantly different from zero ($P > 0.90$).

The correlation of the results of the clostridial assay with those of the colorimetric assay using paired samples yielded a correlation coefficient of 0.994 ($P > 0.95$). The slope of the regression line was 0.963, not significantly different from 1.0 ($P > 0.95$), with a Y intercept of 1.21.

Assays for tetracycline. Assays for tetracyclines, including doxycycline and minocycline, can be performed using the inhibition of hemol-

ysis principle. The regression lines relating tetracycline, doxycycline, and minocycline zone sizes to the $\log_{10}$ concentration of the antimicrobial agent were linear from 0.25 to 32 $\mu\text{g/ml}$. As with chloramphenicol, incubation of the tetracycline assays for greater than 12 to 18 h led to the formation of indistinct zones of macroscopic growth inhibition. However, this observation did not interfere with the assay, since a briefer period of incubation was routinely used.

Assay for penicillin G. Differences between bacteriostatic and bactericidal antimicrobials were noted with this assay system. With penicillin G as the prototype bactericidal agent, clearly defined zones of inhibition of hemolysis were noted at 2 h. Further incubation led to a diminution of the zone of intact erythrocytes, followed by formation of well-defined zones of growth inhibition at 12 to 24 h. Since the zones of intact erythrocytes appeared to be more prone to disintegration as compared to those formed by bacteriostatic agents, the assay for penicillin G required frequent observation to note the formation of the early zones. The transient nature (1 to 2 h) of the intact erythrocyte zone is a disadvantage for rapid readings. The most convenient method of assaying penicillin with this system is by overnight incubation to form stable zones of growth inhibition.

The regression line relating serum concentration to zone size was linear from 1 to 128 $\mu\text{g/ml}$ for both the inhibition of hemolysis zones and the zones formed by overnight incubation. Penicillin levels as low as 1.0 $\mu\text{g/ml}$ are detectable by this method.

Assays in the presence of gentamicin. The addition of 64 μg of gentamicin per ml to the assay medium did not influence the assays for chloramphenicol or the tetracyclines (Table 1). Assays for penicillin G at 1.5 to 3 h and at 24 h with and without gentamicin present compared favorably with those obtained using the *B. globigii* method (Table 2).

TABLE 1. Comparison of assays for chloramphenicol and tetracycline with and without 64 μg of gentamicin per ml using *C. perfringens*

Assay	Sample	Concn ($\mu\text{g/ml}$)	
		With gentamicin	Without gentamicin
Tetracycline	1	1.6	1.7
	2	2.9	2.7
	3	4.1	3.9
Chloramphenicol	1	2.3	2.5
	2	8.5	8.3
	3	19.0	18.5

TABLE 2. Comparison of *C. perfringens* and *B. globigii* assays for penicillin G

Method	Time (h)	Penicillin G sample (μ g/ml)			
		1	2	3	4
<i>C. perfringens</i>	1.5-3	7.4	19.5	26.5	66.0
	24	6.8	18.5	22.5	65.0
<i>C. perfringens</i> with 64 μ g of gentamicin per ml	1.5-3	7.4	19.0	25.0	64.0
	24	7.2	20.5	26.0	66.0
<i>B. globigii</i>	24	8.0	20.0	24.0	68.0

DISCUSSION

The utilization of hemolysis as a biological indicator of microbial growth in antibiotic assays was originally proposed by Sir Alexander Fleming (4). Other investigators have since applied this principle to assays for penicillin and erythromycin using a B-hemolytic streptococcus as the test organism (5). However, this method has not attained any degree of popularity.

Renewed interest in this principle has been kindled by employing *C. perfringens* as the assay organism because of its rapid growth and the elaboration of a potent lecithinase, the alpha toxin. Levison (10) and Sabath and Toftegaard (14) have recently reported the use of *C. perfringens* in assays for metronidazole and clindamycin, respectively. The uniform resistance of *C. perfringens* to gentamicin was noted as a specific advantage by these authors in testing serum in which gentamicin was concurrently present. The current vogue of antimicrobial combination therapy in many infections makes this method particularly attractive in assessing the levels of penicillins, tetracyclines, and chloramphenicol. Determination of cephalosporins is also feasible with this method.

The resurgence of interest in chloramphenicol for *H. influenzae*, intraabdominal and central nervous system infections has made a reliable, rapid method of measuring serum levels highly desirable. This agar diffusion assay, based on the inhibition of hemolysis by the antimicrobial agent, produced consistent results in a short time. Bacteriostatic drugs are easily assayed with this method, overcoming the disadvantages of previous procedures. In addition, bactericidal drugs can be assayed with results comparable to those of conventional methods.

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