Rapid Gentamicin Bioassay Using a Multiple-Antibiotic-Resistant Strain of *Klebsiella pneumoniae*

MARLYS E. LUND, DONNA J. BLAZEVIC, AND JOHN M. MATSEN

Departments of Laboratory Medicine and Pathology, Microbiology, and Pediatrics, University of Minnesota Hospitals, Minneapolis, Minnesota 55455

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A rapid bioassay for gentamicin levels in serum was developed by using a strain of *Klebsiella pneumoniae* that has multiple resistance to antibiotics. Assays were comparable when performed on either small or large petri plates, and results were available in 2 to 4 h. Studies showed an overall recovery of 97.6% for gentamicin alone or 104.5% in the presence of commonly used antibiotics. The procedure can be performed without the need to inactivate other antibiotics that may be present in the serum sample.

Several studies have now been reported which define methods for the rapid assay of gentamicin in body fluids (1, 6, 9, 10, 12, 13, 19, 20-25). The bioassay methods use a test organism susceptible not only to gentamicin but also to many antibiotics commonly used therapeutically in combination with gentamicin, thus requiring some procedure for the inactivation of these other antibiotics when this is possible. The other methods necessitate working with labeled substrate or with labeled gentamicin and antibody against gentamicin. Each of these methods either requires a sophistication beyond the scope of many laboratories or presents limitations which prevents its use in patients in certain clinical circumstances.

We report here a method of assay employing a stable, multiple-antibiotic-resistant *Klebsiella pneumoniae*. This method permits the rapid assay of gentamicin in most patients being treated with gentamicin alone or in combination with other antibiotics. The method eliminates the need for inactivation of accompanying antibiotics and reagents not commercially available.

MATERIALS AND METHODS

Organism used in preparation of assay plates. The strain of *Klebsiella* (University of Minnesota, K-1296) used to seed the agar assay plates was originally isolated in April 1968 and has been used to assay serum gentamicin levels since August 1971. Biochemical identification of the organism was performed by using the methods outlined by Edwards and Ewing (5). The antibiotic susceptibility pattern

of *Klebsiella* 1296 was determined both by the disk diffusion method of Bauer, Kirby, Sherris, and Turck (2) and by minimal inhibitory concentrations (MICs) by using a standardized twofold dilution technique in Mueller-Hinton broth by employing an inoculum of 10^6 to 10^8 organisms per ml. The *Klebsiella* 1296 serotype was determined by the methods described by Edwards and Ewing (5).

Preparation of assay plates. Antibiotic medium no. 11 (Difco) was adjusted to pH 7.9 ± 0.1 , dispensed into 25-ml amounts, and autoclaved. The samples were stored at 4 C for up to 1 month. The appropriate number of tubes of agar were melted and cooled to 50 C as needed for the assay of gentamicin. A 0.4-ml sample of a well-mixed overnight Trypticase soy broth (BBL) culture of *Klebsiella* 1296 was added to 25 ml of melted, cooled agar. Alternately, 0.4 ml of a heavily inoculated 5-h broth culture could be used to seed the agar. The inoculum contained on the average 1.72×10^9 colony-forming units (CFU) per ml (overnight cultures: mean = 1.79×10^9 CFU/ml; 5-h cultures: mean = 1.61×10^9 CFU/ml) as determined by 10 successive replicate counts. For the assay of gentamicin in a single specimen, 9 ml of the well-mixed, seeded agar was pipetted into each of two 100 by 15 mm plastic petri dishes. When several assays were to be performed, 25 ml of the seeded agar was poured into each of two 150 by 15 mm plastic petri dishes. After the agar hardened, plates were stored at 4 C and were used on the day they were prepared or on the following day. Plates stored for longer periods of time required prolonged incubation for zones of inhibition to appear. (Plates stored for 2 days required up to 6 h of incubation).

Preparation of stock antibiotics and standards. A 200 μ g/ml gentamicin (Schering Corporation) stock solution was prepared in sterile, distilled water, dispensed into small portions, and frozen at -20 C.

Working standards of 20.0, 10.0, and 2.0 $\mu\text{g/ml}$ were prepared by diluting the stock solution in sterile, single-donor, human serum previously screened for lack of activity against *Klebsiella* 1296. No anti-*Klebsiella* activity has been revealed to date. The working standards were stored at 4°C and were used for a period of up to 2 weeks.

Assay procedure. For each assay, 0.02-ml portions of patient serum were pipetted with sterile, disposable, capillary pipettes (Dade) or with an Eppendorf pipette onto each of four paper disks (0.25 in., no. 740E, Schleicher & Schuell Co.) in a sterile petri dish. Antibiotic working standards were similarly pipetted onto four disks each. The disks were then placed in pairs on duplicate assay plates (8 disks per plate), with members of the same pair placed directly opposite one another. Disks placed on the plates before pipetting absorbed moisture from the agar; this affected reproducibility adversely. When only one assay was to be performed, small plates were used. When several assays were to be performed, large assay plates were used, with up to five patient specimens and three standards per plate (16 disks). Plates were incubated for 4 h at 35°C. Zones were usually apparent within 2 h, but were better defined at 4 h. Zone diameters were measured with either a Fisher-Lilly zone reader or a vernier calipers (Helios) and were recorded to the nearest 0.1 mm. After averaging the zone diameters obtained, a dose response curve was plotted on semilog graph paper by using point-to-point connection of the three standard points. The patient serum gentamicin concentration was estimated from the curve and reported to the nearest 0.1 $\mu\text{g/ml}$.

Standard curve. To determine the linearity of its response to gentamicin, *Klebsiella* 1296 was tested by using the above assay procedure and the following concentrations of gentamicin: 2.5, 5.0, 7.5, 10.0, 15.0, and 20.0 to 100.0 $\mu\text{g/ml}$ in 10 $\mu\text{g/ml}$ increments.

Recoveries. To determine the ability of the assay procedure to recover gentamicin, known amounts of gentamicin were added to human sera. These sera were then assayed for gentamicin by using the *Klebsiella* 1296 bioassay, and the percentage of recovery of the added amount was determined. The recovery, then, is a measure of the method's accuracy.

Recoveries were performed by adding 0.02 ml of concentrated stock solutions of gentamicin in sterile, distilled water (500, 400, 300, 200, and 100 $\mu\text{g/ml}$) to 0.5 ml of human serum. Twenty-five recoveries using large assay plates were performed on normal, single-donor human serum, and another 25 were performed on serum taken from a Gambro dialyzer. An additional 10 recoveries by using small plates were performed on patients' sera on which a gentamicin assay had been requested.

Recovery of gentamicin in the presence of other antibiotics was tested by adding 0.01 or 0.02 ml of concentrated antibiotic to 0.5 ml of serum containing 10.0 μg of gentamicin per ml. Ampicillin was tested at a final concentration of 200 $\mu\text{g/ml}$. Carbenicillin, cephalothin, chloramphenicol, clindamycin, kanamycin, methicillin, penicillin, and tetracycline were tested at a concentration of 100 $\mu\text{g/ml}$. In addition, cephalothin was tested at 300 and 500 $\mu\text{g/ml}$ and carbenicillin and penicillin at 1,000 $\mu\text{g/ml}$.

All recovery specimens were assayed by using the routine procedure without modification.

RESULTS

Klebsiella 1296 is a biochemically typical *K. pneumoniae* with the exception of its negative reaction on Christensen's urea. It is a serotype 30 and is resistant to most commonly used antibiotics. Table 1 presents the MICs of *Klebsiella* 1296 with 16 commonly used antibiotics and the corresponding zone diameters obtained in the disk diffusion antibiotic susceptibility test. The susceptibility pattern of *Klebsiella* 1296 (susceptible only to gentamicin, colistin, polymyxin B, and naladixic acid) has remained stable since 1968 despite repeated subculturing. A check for reversion to susceptibility was performed routinely by doing a disk diffusion susceptibility test in parallel with the gentamicin assay anytime we received a specimen from a patient on combination antibiotic therapy.

The response of *Klebsiella* 1296 to gentamicin under the conditions of the assay procedure presented is linear up to a concentration of 20 $\mu\text{g/ml}$ (Fig. 1). This allows the use of undiluted patient serum in the assay whether one expects therapeutic or toxic levels.

Table 2 presents the results of 60 recoveries, 50 performed on large assay plates with up to five specimens per plate and 10 on small plates with one specimen per plate. The overall recovery rate for the large plates was 95.7%, whereas that for the small plates was 98.6%. This

TABLE 1. Susceptibility of *Klebsiella* 1296 to 16 antibiotics

Antibiotic	MIC ^a	Zone size ^b
Ampicillin	> 1,000	6
Carbenicillin	> 2,000	6
Cephalothin	300	6
Chloramphenicol	> 2,000	6
Clindamycin	500	6
Colistin	3.1	14
Erythromycin	800	8
Gentamicin	1.5	20
Kanamycin	> 1,000	6
Methicillin	> 1,000	6
Naladixic acid	12.5	16
Nitrofurantoin	500	7
Penicillin G	> 2,000	6
Polymyxin B	1.5	15
Streptomycin	> 1,000	6
Tetracycline	> 2,000	6

^a Minimal inhibitory concentration in $\mu\text{g/ml}$ (U/ml for penicillin G and polymyxin B).

^b Inhibition zone diameter in millimeters by the disk diffusion method.

difference is not statistically significant. The overall mean errors for large and small plates, respectively, were -0.6 and $0.0 \mu\text{g/ml}$. Although the overall range of error appears large (-3.2 to $+1.7 \mu\text{g/ml}$), the large errors occurred in specimens at the extremes of the range of gentamicin concentrations tested, especially at very high concentrations. The difference seen in recovery rates for concentrations greater than $12 \mu\text{g/ml}$ as opposed to those less than $12 \mu\text{g/ml}$ is statistically significant ($P < 0.001$). In no case would a therapeutic concentration of gentamicin have been reported as either toxic or inadequate. Nor would any toxic or inadequate concentration have been reported as therapeutic.

The recovery studies also revealed a difference between the rate of recovery from the serum from a Gambro dialyzer on the one hand and normal or patient serum on the other. The dialyzer sera recoveries were significantly lower ($P < 0.01$).

None of the antibiotics tested in combination with gentamicin significantly affected the amount of gentamicin recovered. The mean recovery rate for gentamicin in the presence of other antibiotics was 104.5% (mean error $0.4 \mu\text{g/ml}$).

DISCUSSION

There is general agreement on the need for a rapid assay for serum gentamicin levels that can be used routinely in a diagnostic laboratory. A

FIG. 1. Standard curve of *Klebsiella* 1296 with gentamicin in the bioassay.

number of rapid assays are currently available. These fall into three classes—the adenylation radioactive assay (21), radioimmunoassay (9), and bioassay (1, 6, 10, 12, 13, 19, 20, 22–25). The adenylation method and the radioimmunoassay have the disadvantages that (i) they employ reagents not now obtainable commercially and (ii) they require the use of equipment and techniques not available to many laboratories. The bioassays avoid these problems. The bioassays using a measurement of pH changes (6, 12, 13) have the disadvantage of using relatively more serum than do the other bioassays. Even without replicates, 0.4 ml of serum is required in the bioassay using a *Proteus* species in a urea-containing broth (13–15). This requirement may prove critical in pediatric specimens. Furthermore, the *Proteus* bioassay of gentamicin shares the major limitation of some of the disk diffusion assays; it is not applicable in the presence of certain antibiotics (e.g., chloramphenicol and kanamycin) (15). The fully evaluated disk diffusion assays currently available employ organisms susceptible to many antibiotics. Although some of these antibiotics can be inactivated by the appropriate β -lactamase (available from Whatman Biochemicals, Ltd., Springfield Mill, Maidstone, Kent, England), the presence of others (chloramphenicol, kanamycin, clindamycin, tetracycline, and erythromycin) precludes the measurement of gentamicin in serum containing the corresponding combinations of antibiotics. Although other investigators have reported using multiply resistant organisms in the bioassay (1, 17, 18), only Alcid and Seligman have presented data obtained with such an organism to allow evaluation of the method. The recovery studies with *Klebsiella* 1296 indicate the applicability of the method for routine laboratory use.

The recoveries of gentamicin from serum from a Gambro dialyzer point up the need for caution in accepting specimens for gentamicin assay. The temptation to draw postdialysis specimens from the dialyzer tubing rather than

TABLE 2. Recovery of gentamicin on large and small plates

Gentamicin concn ($\mu\text{g/ml}$)	Size of plate (mm)	Type of serum	No. tested	Mean recovery (%)	Mean error ($\mu\text{g/ml}$)	Range of error ($\mu\text{g/ml}$)
> 12	150×15	Normal dialysis	10	94.2	-1.0	$-1.9 + 0.3$
			10	88.1	-2.1	$-3.2 + 0.2$
6–12	150×15	Normal dialysis	10	98.7	0.0	$-1.3 + 0.9$
			10	94.2	-0.6	$-1.7 + 0.9$
< 6	150×15	Normal dialysis	5	109.5	$+0.4$	$0.0 + 0.9$
			5	89.5	-0.5	$-1.3 + 0.9$
7–17	100×15	Patient	10	98.6	0.0	$-1.6 + 1.7$

from the patient is great. The worth of such a specimen is doubtful because recovery of gentamicin will be significantly low. The cause of reduced recoveries in these specimens is not certain. It cannot be due to dialysis, because gentamicin for recovery was added after collecting the specimen. The presence of heparin in the dialyzer blood may be to blame (8) in spite of a recent report (26). Although Yourassowsky et al. report that low levels of heparin do not interfere with the recovery of gentamicin in the bioassay procedure, their data indicate a decrease in zone size of 2 to 3 mm with the presence of 5 U of heparin per ml in the bioassay method of Bennett et al. (3). The pH and ion content (11) of the dialysis bath might also influence the recovery of gentamicin.

The use of a multiply resistant organism avoids many of the disadvantages seen in the currently available bioassays. Most important, a multiply resistant organism such as *Klebsiella* 1296 allows the measurement of gentamicin when it is combined with antibiotics not inactivated by β -lactamase preparations. The growing use of the combination of gentamicin and clindamycin makes this advantage especially important. Furthermore, it is not necessary to use β -lactamase except in rare circumstances. On sera from patients in renal failure who have been on prolonged cephalothin therapy, the use of β -lactamase is recommended, because one can anticipate the possibility of very high serum cephalothin levels in these patients (7, 16). However, even an enzyme preparation with low activity is adequate, because it is not necessary to inactivate all of the cephalothin present.

Dilution of patient serum either with enzyme or as routine procedure is to be avoided if at all possible, especially when dealing with small amounts of specimen. The errors inherent in any bioassay procedure are magnified when results are multiplied by a dilution factor. The dose response of *Klebsiella* 1296 to gentamicin allows the use of undiluted sera in the bioassay.

Whether or not the necessity of carrying a fresh broth culture of *Klebsiella* 1296 is a disadvantage or not depends on one's point of view. The preparation of assay plates from melted, cooled, preportioned agar takes little time. This avoids the problems of running out of plates when the assay workload is heavy or of discarding outdated plates when the workload is light. According to Reeves (17), it is possible, if facilities are available, to store a prepared inoculum frozen in liquid nitrogen.

We feel that it is necessary to run at least a three-point standard line every time an assay is performed. This practice allows for any changes

due to variation in medium composition, either batch-to-batch or lot-to-lot variation. The use of a standard line with each assay also permits variation in the depth of the agar without significantly affecting the results. The agar depth must, of course, be kept as uniform as possible in any one plate, but plate-to-plate variations due to pipetting errors or to variations in the plastic petri dishes are obviated (4). Finally, the inoculum used to seed the assay plates can vary without significantly affecting the results obtained.

The use of 150 by 15 mm plates for multiple assays has proved very helpful in our laboratory. Technologist time both in setting up and reading gentamicin assays has been considerably reduced by reducing the number of standard disks required. Only six standard disks are required for five assays instead of the 30 which would be required if only one specimen could be run per plate. The large plate method should be adaptable to any disk diffusion bioassay.

(*Klebsiella* 1296 is available from J. M. M.)

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