Serum gentamicin assays of 100 clinical serum samples by a rapid 40°C *Klebsiella* method compared with overnight plate diffusion and acetyltransferase assays

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SUMMARY We have compared the results of gentamicin assay of 100 clinical serum samples by a rapid 40°C plate diffusion method, an overnight plate assay at 37°C, and radioactive acetyltransferase methods. The results of assay obtained by both plate diffusion methods agreed closely. There was excellent correlation between the results of acetyltransferase and plate assays provided that human serum gentamicin standards were used for the acetyltransferase assay and turbid sera were excluded. Lipaemic sera were associated with falsely high results by the acetyltransferase method. There was no difference in specificity between the methods when antibiotics other than gentamicin were present. Much less skilled technician time was required to perform the rapid 40°C plate method than the radioactive acetyltransferase method. The 40°C plate method is preferred for routine serum gentamicin assays in our clinical laboratories.

Serum gentamicin assays are frequently necessary to ensure adequate dosage and to avoid toxic levels in patients with impaired renal function. Most laboratories in Britain use a plate diffusion technique for gentamicin assay but the results obtained by this method are often highly misleading (Reeves and Bywater, 1975). Recent studies on factors affecting plate assays may help to standardise the plate diffusion method and improve the reliability of the results obtained (Shanson and Daniels, 1975; Shanson and Hince, 1977). When *Klebsiella* is used as the assay organism plate diffusion assays can be read after 4 to 6 hours as well as after overnight incubation (Reeves, 1972). The *Klebsiella* strain NCTC 10896 is resistant to many antibiotics and is particularly suitable for gentamicin assay (Waterworth, 1973). The speed of assay with this organism can be increased by raising the temperature of incubation to 40°C and using a heavy inoculum surface seeded on to DST medium; assay results are then available after 2½ hours (Shanson *et al.*, 1976).

A rapid radioactive acetyltransferase method has been recommended recently for gentamicin assay.

This method is highly reproducible and very specific (Broughall and Reeves, 1975a and b).

The main purpose of the present study was to compare the results of serum gentamicin assay of 100 consecutive sera from patients receiving gentamicin using the following methods: (1) 2½ hours at 40°C plate diffusion method, (2) 18 hours at 37°C plate diffusion method, (3) radioactive acetyltransferase method using human serum gentamicin standards, and (4) radioactive acetyltransferase method using horse serum gentamicin standards.

The specificity of the four methods was also compared by adding different antibiotics to sera containing gentamicin and then assaying the serum gentamicin by each method.

Material and methods

One hundred serum samples were collected from 40 patients receiving gentamicin. Some of the patients had impaired renal function and many were receiving antibiotics other than gentamicin at the time of assay. Each serum was assayed by the rapid 40°C method and the results were usually communicated to the clinician within 3 hours of collection of the serum sample. Next day the results of overnight plate assay were read and the radioactive acetyltransferase assays were performed.

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PLATE DIFFUSION ASSAYS

Oxoid Diagnostic Sensitivity Agar (DST), CM261, was used for assay at pH 7.4. Thirty millilitres agar were poured into 100 mm square plates which were then levelled. Plates were stored for one to four days at room temperature before use. On the day of use the plates were pre-dried for 30 minutes at the temperature of assay.

Aliquots of an overnight peptone water culture of *Klebsiella edwardsii* var. *atlantae* NCTC 10896 were stored at -70°C and a fresh sample was thawed each week. From this the *Klebsiella* was subcultured onto blood agar and the next day several colonies were emulsified into peptone water. A few drops of the peptone water suspension were added to 100 ml nutrient broth and this was incubated at 37°C overnight. Dilutions in nutrient broth of the overnight culture were used to surface seed plates. For the 40°C assay a 1 in 10 dilution, and for the 37°C assay a 1 in 100 dilution was used. After seeding, the plates were drained well. Each day a fresh subculture of the organism was made onto blood agar and 100 ml nutrient broth was inoculated for overnight culture, as described above.

Plates for 18-hour assay were left at room temperature after seeding, with the lids on, for 15 minutes. Seeded plates for the 40°C assay were dried with their lids off for exactly 30 minutes at 40°C . During this time the blood sample was centrifuged and the serum separated. Immediately after drying 7 mm wells were cut.

No. 5 inactivated horse serum (Wellcome Reagents Limited) was used to prepare standard gentamicin sera containing 1.25, 2.5, 5.0, and 10.0 $\mu\text{g/ml}$ gentamicin. When a high result was expected the test serum was diluted with an equal volume of pooled human normal serum. A previous study showed that the results obtained with this horse serum were the same as those with human serum (Shanson and Daniels, 1975).

Immediately after the wells had been cut they were quickly and completely filled with the sera. Standard and test sera were introduced into each plate in duplicate, and all the wells of one plate were filled before the next was dealt with. Assays at 37°C were incubated in a hot room, and assays at 40°C in a 40°C incubator. Gentamicin assays of the sera at 40°C were read after $2\frac{1}{2}$ hours', and at 37°C after 18 hours' incubation. The inhibition zone sizes were measured on a zone reader (Leebrook Instruments Co Ltd).

ACETYLTRANSFERASE METHODS

The basic acetyltransferase method used has been described elsewhere (Broughall and Reeves, 1975a). Assays were performed in Cooke microtitre plates

with flat-bottomed wells and incubated for 30 minutes at 37°C . After incubation 70 μl of each assay solution was pipetted onto a 2.5×2 cm rectangle of P81 paper, which was washed in three changes of 5 mm Tris-HCl, pH 7.4 buffer, and finally in distilled water. The papers were dried under an infrared lamp placed in scintillation fluid and counted in an Inter-technique SL30 liquid scintillation counter.

Each test serum was assayed using the same set of horse serum gentamicin standards that were used for plate diffusion assays and also with a set of human serum gentamicin standards.

PREPARATION OF SERA CONTAINING MIXTURES OF ANTIBIOTICS

A pooled normal human serum sample was prepared containing 3.6 $\mu\text{g/ml}$ gentamicin. This sample was divided into several aliquots, to each of which was added a second antibiotic. The second antibiotic was either carbenicillin, 100 $\mu\text{g/ml}$, lincomycin, 30 $\mu\text{g/ml}$, tetracycline, 4 $\mu\text{g/ml}$, cephradine, 25 $\mu\text{g/ml}$, cefazolin, 25 $\mu\text{g/ml}$, or a mixture of trimethoprim, 5 $\mu\text{g/ml}$, and sulphamethoxazole, 100 $\mu\text{g/ml}$.

Results

STANDARD CURVES OF PLATE DIFFUSION AND ACETYLTRANSFERASE METHODS COMPARED USING TWO TYPES OF SERUM FOR GENTAMICIN STANDARDS

With the plate diffusion method the standard curves were virtually identical whichever type of serum was added, but with the acetyltransferase method lower counts per minute were obtained with horse serum than with human serum (Fig. 1).

GENTAMICIN ASSAY OF CLINICAL SERUM SAMPLES BY THE DIFFERENT METHODS

The results of gentamicin assay by the four methods are shown in Fig. 2. Good correlation was obtained between the results of the $2\frac{1}{2}$ -hour plate diffusion method and those of the acetyltransferase method that used human serum gentamicin standards ($r = 0.92$, slope = 1.12, intercept = -0.48). The results of the $2\frac{1}{2}$ hours at 40°C and 18 hours at 37°C plate diffusion assays agreed closely ($r = 0.98$, slope = 0.99, intercept = 0.01). Only four out of 100 serum gentamicin assays were associated with a difference in result of more than 25% between the two plate assay methods, and the greatest difference was 33%. The acetyltransferase assay using horse serum gentamicin standards gave results about 20% higher than those obtained when human serum standards were used. The results of acetyltransferase assay with horse serum standards showed only moderately good correlation with those of plate assay ($r =$

Fig. 1 Comparison of standard curves using human (—) and horse (---) serum gentamicin standards by the acetyltransferase (A) and 18 h plate diffusion (B) methods.

Fig. 2 Comparison of results of 100 clinical serum gentamicin assays by (A) two plate diffusion methods, and (B) two acetyltransferase methods using either human (●) or horse (○) serum gentamicin standards with the 2 1/2 h plate diffusion method.

0.90, slope = 1.25, intercept = 0.20). With eight out of the 100 gentamicin assays of clinical serum samples there was a difference in result of greater than 35% between the 2 1/2 hour plate diffusion assay and the acetyltransferase method using human serum gentamicin standards. Most of these sera were very turbid or haemolysed.

The results of assay of sera which were grossly turbid are shown in Table 1. Overnight plate diffusion assays at 37°C gave results within 0.2 $\mu\text{g/ml}$ of those obtained with the rapid 40°C plate assay. The acetyltransferase method using horse serum standards gave higher results than those observed with the same method using human serum stan-

dards. In every instance the highest results were achieved with the acetyltransferase methods. The fluorimetric assay method, performed independently at another hospital, gave results which correlated closest with the plate diffusion method.

COMPARISON OF THE ANTIBIOTIC SPECIFICITY OF THE PLATE DIFFUSION AND ACETYLTRANSFERASE ASSAYS

Table 2 shows the result of assay of a serum containing gentamicin alone compared with the same serum containing one other antibiotic in addition. Both the plate diffusion and acetyltransferase methods appeared to be specific for gentamicin in the

Table I Results of gentamicin assay of five grossly lipaemic sera by different methods ($\mu\text{g/ml}$)

Serum No.	Assay method		
	2½ h at 40°C plate diffusion	Radioactive* acetyltransferase	Automated* fluorimetric†
8	2.4	3.0	1.7
14	8.9	13.0	9.7
13	6.7	9.3	5.2
15	10.0	15.0	8.9
10	4.5	8.4	5.7
Mean and standard deviation	6.5 $\pm$ 3.1	9.7 $\pm$ 4.6	6.2 $\pm$ 3.2

*Using same human serum gentamicin standards.

†Independently performed at another hospital.

Table 2 Results of gentamicin assay by plate diffusion and acetyltransferase methods when other antibiotics were present

Serum	Plate assay result (mg/l)		Acetyltransferase result	
	2½ h/40°	18 h/37°	Human serum gentamicin standard	Horse serum gentamicin standard
Gentamicin 'X' (mg/l)	3.1	3.6	3.6	4.2
Gentamicin 'X' (mg/l) + Lincomycin, 30 mg/l	3.2	3.2	3.1	3.5
Gentamicin 'X' (mg/l) + Cefazolin, 25 mg/l	3.4	3.9	3.4	3.9
Gentamicin 'X' (mg/l) + Cephadrine, 25 mg/l	3.1	3.6	3.6	4.1
Gentamicin 'X' (mg/l) + Tetracycline, 4 mg/l	3.6	3.3	3.8	4.3
Gentamicin 'X' (mg/l) + Carbenicillin, 100 mg/l	3.0	3.2	3.4	3.9
Gentamicin 'X' (mg/l) + Trimethoprim, 5 mg/l, and Sulphamethoxazole, 100 mg/l	3.2	3.3	3.5	4.0
Mean $\pm$ SD	3.22 $\pm$ 0.20	3.44 $\pm$ 0.26	3.48 $\pm$ 0.21	3.98 $\pm$ 0.26

presence of either carbenicillin, cotrimoxazole, tetracycline, cephradine, cefazolin, or lincomycin in the concentrations stated in the table.

Discussion

The results of clinical serum gentamicin assay by the rapid 40°C plate method agree closely with those of overnight plate assay. Good correlation between these plate diffusion methods has been observed previously in a study using simulated test sera (Shanson *et al.*, 1976).

There was good correlation between the plate diffusion and acetyltransferase methods using human serum gentamicin standards provided that very turbid or haemolysed sera were excluded. All the turbid sera examined in this study were grossly lipaemic and were from two patients. One patient received intralipid intravenously but the other patient had no parenteral lipid therapy. The latter patient was an obese alcoholic patient who was on the intensive care unit. Higher results were obtained with these lipaemic sera by the acetyltransferase assay compared with the plate and fluorimetric assay methods. It has been suggested that lipids coat the phosphocellulose paper used in the acetyltransferase method, making it difficult for any unreacted radio-

active labelled C^{14} acetylcoA (Broughall and Reeves, 1975b) to be washed away completely, and that this method is contraindicated in patients receiving intralipid. No previously published data are available showing how much the results are affected by turbid sera.

Unlike plate diffusion assay the horse serum gentamicin standards gave different standard curves for the human serum standards with the acetyltransferase assay. This resulted in higher values of serum gentamicin from test sera when horse rather than human serum standards were used. Only human serum gentamicin standards can be recommended for the radioactive enzyme assay if the results are expected to correlate closely with those of plate diffusion assay.

Rapid gentamicin assays have the advantage that advice on the next dose of gentamicin can be given on the same day that the serum samples are received. On a Saturday morning and on other days when serum samples are not received until after the middle of the day methods that can give a result in under 3 hours are to be preferred. Most laboratories do not have access to a liquid scintillation counter and the 40°C plate method can then be recommended instead. The radioactive method is particularly suitable for small samples which may be

relevant in neonatal or paediatric practice. The enzyme method can also be quicker as results are available after 1½-2 hours compared with 2½-3 hours for the 40°C plate assay. In practice, a result which is available after 3 hours is usually fast enough. Once the radioactive enzyme method has been in use for a short time it is not too difficult to achieve reliable results. In contrast, a more experienced and skilled technician is needed to perform reliable plate assays.

The main advantages offered by the 40°C plate assay over the acetyltransferase assay are simplicity, cheapness, safety, and, most important, a considerable saving in skilled technician time. The total amount of skilled technician time required to perform serum gentamicin assays on a couple of serum samples by the acetyltransferase method is about 45 minutes compared with about 5 minutes for the rapid plate assay.

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