Technical methods

Rapid reading and recording of complement fixation tests by means of a microtitre plate scanner

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Selection of microbial antibody tests according to the clinical features of the patient's illness has the obvious advantages of medical appropriateness and economy, which will appeal to many clinical serologists. It presupposes, however, that sufficient clinical information is either provided or readily obtainable and that translation of these data into a small number of antibody tests will follow without delay. Unfortunately, both these suppositions are becoming increasingly unrealisable. Many laboratories, therefore, resort to a type of testing whereby each serum is run against an extensive panel of antigens, irrespective of clinical indication. One advantage of this system is that clinical features not hitherto associated with certain agents may be recognised. This approach has been facilitated by the availability of multichannel dispensers, which have greatly offset the increased technical work involved. Unfortunately, the work of visually reading and recording the increased number of tests generated by this means has now become a problem of considerable dimensions. We have therefore investigated the feasibility of reading and recording tests by a rapid, non-subjective means. The machine used (Fig. 1) was a Titertek Multiskan Plate Reader (Flow Laboratories, Irvine, Scotland). This electronic apparatus, although designed for enzyme-linked immunosorbent assay, has been employed in our study to read and record complement fixation (CF) tests.

Material and methods

PLATE READER

The machine is an eight-channel photometer and has an output terminal for a computer interface. It automatically reads simultaneously the contents of a column of eight wells through a microtitre plate, and all 96 readings are printed out within a minute on to paper tape. A 450 nm optical interference filter was

selected for the work described here. Specification and operating instructions are provided with the apparatus.

MICROTITRE PLATES AND READINGS

Rigid polystyrene plates with 96 wells were used. A horizontal line of wells is termed a 'row' and a vertical line a 'column'. The rows and columns are permanently labelled.

The readings were (a) conventional visual recordings of red cells settled in round base wells, and (b) optical density readings made in the machine on cells *in suspension* in flat base wells (Linbro, Flow Laboratories).

Fig. 1 Titertek Multiskan Plate Reader.

MICROTITRE PLATE SHAKER

As readings on the machine are made on cells in suspension, individual plates were vibrated on a microtitre plate shaker (Dynatech Laboratories, Billingshurst, Sussex) immediately before recording.

LYSED AND UNLYSED 2% SHEEP RED CELLS

Adjusted concentrations of 2% red cells (Table 1) were prepared for simulating CF readings and for the calibration of the plate reader. Lysate was prepared by immersing a bottle of a 2% suspension of red cells in an ultrasonic cleaning bath (Polaron Equipment, Watford, Herts).

Table 1 *Adjusted concentrations of unlysed and lysed 2% sheep red cells*

Cell and lysate	% cells	% lysis
(a) lysate*	Nil	100
1 part (b) + 3 parts (a)	25	75
1 part (b) + 1 part (a)	50	50
3 parts (b) + 1 part (a)	75	25
(b) 2% sensitised cells	100	Nil

*2% lysed sensitised cells.

VIRUS AND OTHER ANTIGENS

Influenza A and B, adenovirus, para influenza type 3, measles, mumps S and V, herpes simplex, psittacosis, Q fever phase 2, and *Mycoplasma pneumoniae*.

DILUENT

Barbitone diluent code Br. 16 (Oxoid, Basingstoke, Hants).

CALIBRATION OF PLATE READER

A blanking operation is performed on a column of eight wells containing 25 μ l lysate and 75 μ l diluent (when antigen, complement, or specimen is not represented, a replacement volume of diluent is

added to the sample to be tested). This solution must give zero reading (Table 2a). In the next operation

Table 2 *Multiskan readings on adjusted concentrations of 2% red cells: Example*

	Rows				
	E	D	C	B	A
(a) (Blank on lysate)					
Readings	0	0	0	0	0
(b) (% cells)	100	75	50	25	0
Readings	9	8	5	3	0

In (b) the range index was 0.9.

with the machine in the 'matrix mode', a suitable range index is selected. An example of the readings of different concentrations of red cells for a 'range' index of 0.9 on this occasion is shown in Table 2b.

REAGENT DISPENSING

The reagents for the diagnostic tests were dispensed with a 96-channel automatic pipetter (Dynatech Laboratories). For the other tests, reagents were dispensed by means of a hand-held automatic pipette.

Table 3 *Reproducibility: Adjusted concentrations of 2% red cells in 10 columns of wells*

Columns						Rows					
						E	D	C	B	A	
1-10						100	75	50	25	0%	

Column	Readings I-VII					Column	Reading VIII				
	Rows						Rows				
	E	D	C	B	A		E	D	C	B	A
1	9	7	5	3	0	1	9	7	5	3	0
2	9	7	5	3	0	2	9	7	5	3	0
3	9	8	6	3	0	3	8	7	6	3	0
4	9	8	5	3	0	4	9	8	5	3	0
5	9	8	6	3	0	5	9	8	6	3	0
6	9	7	5	3	0	6	9	7	5	3	0
7	9	7	5	3	0	7	9	7	5	3	0
8	9	7	5	3	0	8	9	7	5	3	0
9	9	7	5	3	0	9	9	7	5	3	0
10	9	7	6	3	0	10	9	7	6	3	0

Reading IX						Reading X					
1	9	7	5	3	0	1	9	7	5	3	0
2	9	7	5	3	0	2	9	7	5	3	0
3	8	7	6	3	0	3	8	7	6	3	0
4	9	7	5	3	0	4	9	8	5	3	0
5	9	7	6	3	0	5	9	7	6	3	0
6	9	7	5	3	0	6	9	7	5	3	0
7	9	7	5	3	0	7	9	7	5	3	0
8	9	7	5	3	0	8	9	7	5	3	0
9	9	7	5	3	0	9	9	7	5	3	0
10	9	7	5	3	0	10	9	7	6	3	0

Table shows a plate with actual cell concentrations and results of 10 replicate readings.

Readings I-VII show concordant results, and readings VIII, IX, and X show the discordant results in bold type.

Results

TESTS OF REPRODUCIBILITY OF THE PLATE READER

After completion of the blanking and calibration operation 10 replicate readings were made on 10 columns of wells in a single plate. Of the 500 readings, 10 were discordant (Table 3).

TESTS TO DETERMINE INFLUENCES OF RED CELL CONCENTRATIONS ON READINGS

Two columns of wells in a plate were filled with alternate high and low concentrations of red cells. Readings (Table 4) showed no evidence of interference due to cell concentration.

Table 4 *Influence of variation of red cell concentration on optical density readings*

% Concentration	100	0	100	25	100	50	100	75
Readings	9	0	9	3	9	5	9	7
% Concentration	0	100	25	100	50	100	75	100
Readings	0	9	3	9	5	9	7	9

EFFECT OF REAGENTS ON MULTISKAN READINGS

As the machine detects changes in optical density it was considered necessary to investigate the effect of various standard reagents used in CF tests in respect

of any variation in test results. Different concentrations of sensitised cells (Table 1) and other reagents (prepared for routine use) were added to wells in a microtitre plate. To prevent cell lysis by antigen, antibody, and complement interaction (when all the reagents are mixed together), 1% formalin was added to the cells. The serum, complement, and antigens (at working dilutions) individually, or in combination, did not influence readings across the range of cell suspensions.

INHERENT VARIATIONS IN OBSERVER READINGS ON RED CELLS SETTLED IN ROUND BASE WELLS

As comparisons of machine readings would ultimately be made with results obtained by visual readings, an assessment of the reproducibility of the latter was also made. The predictions of three observers on a series of prepared concentrations of red cells are shown in Table 5. Observers A and C recorded 50% cells for all wells having 25% cells. Observer B also recorded the same higher value for 14 of the 15 wells with 25% cells, and observers A and B also recorded all wells with 50% cells as having 75%. Inability to detect the true value of the lower concentrations is clearly demonstrated, the observers all reading high. The results of a further experiment are shown in Table 6. A series of con-

Table 5 *Readings by three observers (A, B, and C) on settled red cells in a microtitre plate*

Prediction	Adjusted concentration of cells								
	25% (15)			50% (7)			75% (11)		
	A	B	C	A	B	C	A	B	C
25%	0	1	0						
50%	15	14	15	0	0	7			
75%				7	7	0	11	11	11
100%								14	14

Number of wells containing the concentration in parentheses.
Correct readings in bold type.

Table 6 *Predictions of 25% cell concentrations by four observers on randomised cell concentrations in a microtitre plate*

25% Predictions	Adjusted concentration of cells						Predictions of '25% value'
	10% (5)	15% (9)	20% (9)	25% (14)	30% (17)	35% (9)	
Observer A	5	1	0	0	0	0	6 (nil correct)
Observer B	0	6	3	2	1	0	12 (2 correct)
Observer C	0	3	16	13	8	0	40 (13 correct)
Observer D	2	8	4	0	0	0	14 (nil correct)

Number of wells containing the concentration in parentheses.
Correct predictions in bold type.

centrations (around a mean of 25% concentration) was randomly distributed in a microtitre plate, and observers were asked to identify all wells which they thought contained the 25% concentration. This concentration was in fact present in 14 of the 63 wells. Again the inability of observers to identify the lower cell concentrations is apparent. This inherent variation in visual predictions by different observers can also be seen in the readings made on a batch of routine clinical tests (Table 7). Whatever the

Table 7 Variations in visual readings on a batch of CF tests: readings by three observers (A, B, and C)

Residual cells	Observer			No. agreed by A, B, C
	A	B	C	
25%	9	7 (4)	23 (8)	4
50%	7	20 (4)	12 (5)	3
75%	21	31 (13)	23 (13)	5
100%	51	23 (23)	42 (42)	21

No. of readings in agreement with observer A in parentheses.

'correct' answer may have been, very little agreement could be shown between the readings of the different observers. Thus the comparison of the readings on the plate reader with visual observations presents some degree of difficulty as the latter readings are somewhat a matter of arbitrary choice. This element of subjectivity in conventional readings has to be taken into account when machine and visual readings are compared.

CORRELATION BETWEEN CF ANTIBODY TITRES DETERMINED BY VISUAL AND MULTISKAN READINGS

In order to make comparisons between the two methods of reading CF tests, specimens of sera submitted for routine clinical investigation were titrated in parallel against the antigens already listed above. A conventional visual end-point reading was defined as the highest dilution of serum showing 50% or more red cells settled in a round base well.¹ For obtaining results on the machine, plates with flat-base wells were used. The numeral for 50% residual cells was obtained by means of an adjusted suspension of cells (Tables 1 and 2), and an end-point reading was the highest dilution of serum giving this numerical value or higher. Of the 368 titrations examined in duplicate, 132 end-points were less than the first dilution (1 in 10) in both reading systems. Of the remaining 236 titrations, 118 were identical, 110 had results one dilution higher when read by the visual method, whereas only six gave readings 1 dilution lower when read by this method. Two visual readings gave higher end-points by two dilutions. As shown in Fig. 2, the bias towards higher visual

Fig. 2 Correlation between CF antibody titres determined by visual readings and Multiskan readings.

recordings was distributed throughout the dilution series. This was predictable from the data shown in Tables 5 and 6. Readings with true values of 50% residual cells or more would not be affected by the different methods of recording, as both would give the same dilution as the end-point. However, where the true value in a dilution was less than 50% cells the machine would record the dilution below as the end-point (by definition) but there would be a tendency by the visual reader to over-read the cells and, in a proportion, record these as 50% or more, thus reading one dilution higher than the machine reading.

Discussion

Laboratories providing clinical pathology services are labouring under an ever increasing workload.² In an effort to find a means of alleviating some aspects of this problem in clinical serology we have assessed the Multiskan Plate Reader.

This machine is capable of reading and recording large batches of serological tests, producing a print-out of the results. To deal with comparable batches manually usually entails two workers, one to read and another to record. Its operation on each 96 well microtitre plate is completed within a minute with no apparent risk of transcription error. Furthermore, readings from the machine are made on cells in suspension, whereas the visual method

requires extra time for cells to settle before readings are made.

In our hands, this equipment has proved reliable and simple to use, and recordings have been reproducible and free from the bias detected in results derived from human observation.

Although the Multiskan Plate Reader was designed for enzyme-linked immunosorbent assay, its role in reading CF tests in the manner described above has proved satisfactory, and it is possible that it will prove satisfactory for other types of serological tests also. It has the advantage that it reads the microtitre plates directly, thus avoiding the need for transfer of the well contents to cuvettes. The plate reader has a facility for computer linkage, and a fully automated system of serological testing and reporting could be developed.

We are indebted to Mrs Sheila Holt and Miss Susan Jordan for technical assistance. We also wish to thank Flow Laboratories for the use of the plate reader.

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Influence of clearing agent on immunohistochemical staining of paraffin-embedded tissue

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It is well recognised that fixation is an important factor in the determination of immunoreactivity of antigens in paraffin-embedded tissues.¹⁻⁴ The effect of subsequent tissue processing has rarely been studied^{5,6} and the ethanol-chloroform schedule appears to be the 'routine method' when details are available.^{7,8} Many laboratories use xylene as their routine clearing agent, which has been shown to have an extracting quality on cell cytoplasm as seen ultrastructurally⁹ and to decrease immunoreactivity of frozen sections.⁶ This paper describes a study to determine the effect of different clearing agents on subsequent immunoreactivity of fixed and embedded tissue.

Material and methods

Slices of palatine tonsil 2-3 mm thick were fixed for 4 or 22 hours at room temperature in the fixatives listed (Table), processed via ethanol (12 hours) and xylene, chloroform or Inhibisol (methyl chloroform containing a patented inhibitor—Bestobell Chemical Products Ltd, Mitcham, Surrey)¹² at room temperature (10 hours) and waxed at 60° (3 hours); 5 μ sections were mounted on clean, adhesive-free slides and dried at 56° for 2 hours. Before immunostaining, sections were dewaxed in xylene (3 $\times$ 1 min) and rehydrated through 74 OP ethanol (3 $\times$ 1 min) and 64 OP ethanol (1 $\times$ 2 min) to 0.05 M Tris-HCl buffered saline, pH 7.6 (TBS), which was used for trypsinisation and reagent dilution.

B-5 fixed sections were treated with Lugol's iodine (5 minutes) and sodium thiosulphate (0.2 M, 5 minutes) after the diaminobenzidine reaction and before counterstaining. Removal of picric acid by pretreatment of Bouin's fixed tissue with lithium carbonate (saturated solution in 70% ethanol, 2 minutes) did not affect immunoreactivity.

The unlabelled antibody enzyme method (PAP) was performed with and without prior trypsinisation of sections, essentially as described by Curran and Gregory¹³ except that digestion was carried out at room temperature and endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide