COUNTERIMMUNOELECTROPHORESIS AS A ROUTINE MYCOSEROLOGICAL PROCEDURE

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Abstract

Counterimmunoelectrophoresis (CIE) has been compared in a diagnostic laboratory with agar gel double diffusion (DD) as a routine procedure for detection of antibodies to pathogenic and allergenic fungi and actinomycetes. It was shown to be of particular value in detecting antibodies to Aspergillus fumigatus. Thus 72 of 106 sera in which precipitins were detected were positive by CIE alone.

Some sera were positive only by CIE to antigens prepared from Histoplasma capsulatum, Allescheria boydii, Candida albicans and C. parapsilosis.

Introduction

Counter-immunoelectrophoresis (CIE) is a well-established procedure and is routinely used for the detection of Australia antigen in the serum of patients with hepatitis (Gocke & Howe, 1970). Similar techniques, based on counter migration of antigens and immunoglobulins in an electric field have been used in the mycological laboratory for the detection of antibodies to Micropolyspora faeni, Thermoactinomyces vulgaris and avian antigens and to Aspergillus fumigatus, Histoplasma capsulatum, Candida albicans, Coccidioides immitis and Paracoccidioides brasiliensis (Jameson, 1968; Gordon, Almy et al., 1971; Remington, Gaines & Gilmour, 1972; Gaines & Remington, 1973; Galussio, Friedman & Negroni, 1973; Kleger & Kaufman, 1973; Pappagianis & Shifrine, 1973; Viviani, Pagano & Drouhet, 1973; Ward & Kohler, 1973; Flaherty, Barboriak et al., 1974. A combination of CIE and double diffusion (DD) has been used by Conti-Diaz & Somma-Moreira et al., 1973 to detect antibodies to Paracoccidioides brasiliensis.

The principal advantages of the technique compared with Ouchterlony double diffusion tests are its greater rapidity and sensitivity. This report describes the development and evaluation of CIE as a routine diagnostic procedure in a clinical mycological laboratory.

Materials and methods

The method proposed by Jameson (1968) for detecting very small quantities of antibody in the sera of patients with Farmer’s Lung was in use in the Mycological Reference Laboratory (MRL) for some 2–3 years, in parallel with a routine conventional Ouchterlony test. Although sensitive, it was eventually discontinued because of difficulties in distinguishing between specific and non-specific reactions in tests with antigens from Aspergillus and Candida. Following the reports by Gordon (1971) and Remington et al. (1972) interest in developing the technique and applying it to routine serological screening was resumed. Initially, attention was paid to well sizes and spatial relationships. It was found that the greatest number of positive results was obtained with serum wells 4.5 mm diameter, antigen wells 2.5 mm diameter and edge-to-edge distances of 3 mm. It was also found that replacement of agarose with a mixture of equal quantities of agarose and agar as suggested by Gordon (1971) and used by Kleger & Kaufman 1973 resulted in increased migration of reagents, and improved resolution of the lines of precipitation.
The final procedure adopted is as follows:

Buffered medium
- Barbitone 3.44 g
- Sodium barbitone 7.57 g
- Agar
  - (Oxoid Ionagar No. 2) 5 g
- Agarose 5 g
- De-ionised water to 1000 ml.

Reagents were dissolved by steaming at 100°C for 2 hours and the pH adjusted to 8.2 before use.

**Method**

The basic unit for the tests is a 3" x 2" (76 mm x 51 mm) glass microscope slide, cleaned and pre-coated with molten buffered agar: agarose before use. Three vertical rows of paired wells are accommodated on each slide (fig. 1).

The arrangement of wells and conditions for electrophoresis are such that components from one vertical row of paired wells do not migrate as far as an adjacent vertical row.

Prepared slides are placed on a level surface: 5.5 ml of molten buffered gel were added and allowed to set, giving a layer of 1.4 mm deep. The gel must be homogeneous. This is achieved by pouring the molten gel when still hot. Thickness of the gel is important. If too thin, lines are faint or equivocal. If too thick (2 mm or greater), washing has to be extended and the number of non-specific or equivocal reactions increases.

Wells are prepared using stainless steel cutters of appropriate size and a plastic template that fits closely over the slide without touching the gel surface and allows precise alignment of the cutting procedure. Ease of handling of the slides and an increase in the numbers of sera which can be tested is achieved by accommodating 4 slides in a plastic holder designed for micro-immunoelectrophoresis (Shandon). Each set of 4 slides receives 32 ml of molten gel. Since the gel now extends 10 mm laterally from each slide, application of the wicks prior to electrophoresis is simplified. In addition, the possibility of contaminating wicks with fast migrating molecules is effectively minimised. A larger plastic template was prepared which fitted over the whole assembly and allowed alignment of the 72 pairs of wells. Antigen (10 μl) and serum (20 μl) were added to the wells and the slides placed in an electrophoresis apparatus (Shandon), containing barbitone buffer. A current of 4 v/cm was applied through absorbent lint wicks for 60 or 90 minutes. Slides were read directly in a dark ground illuminator. Immersion of the slides in a washing solution (0.4% sodium tetraborate and 0.4% sodium chloride) for 1 hour (Remington, personal communication) eliminated occasional non-specific reactions. Slides were subsequently returned to the washing solution for an additional 2-3 days, rinsed briefly in tap water, dried in a hot air cabinet and stained with 0.5% Coomassie Brilliant Blue in menthol: acetic acid: water (5:1:4). The time required for removal of unprecipitated protein from the gels was eventually reduced to 24 hours by standing the slide holder on edge, at an angle of 45°, in a wash-bat containing a rotating magnetic stirring bar. Drying of the gels prior to removal of unwanted protein allowed rapid diffusion (1-2 hours) from agarose but this method was not found practicable with an agar agarose mixture.

**Antigens**

Choice of antigens was determined largely by the type of serological tests that this laboratory is routinely asked to undertake. The primary intention was to compare CIE with the standard Ouchterlony double diffusion test. Attention was therefore focussed on the detection of antibodies to Aspergilli, *Candida*, and agents associated with Farmer’s Lung.

Fungal and actinomycete antigens were prepared as follows.

![Fig. 1. Pattern for CIE. Test sera in large wells. Antigens in small wells. Cathode to the left.](image-url)
A. fumigatus (MRL strains 2109, 2140)  
A. terreus (strain 2026)  
A. niger (strain 2022)  
A. nidulans (strain 2020)  
A. flavus (strain 2008)  
Allescheria boydii (strain 1002)  
Histoplasma capsulatum (strain 4037)  
Mieropolyspora faeni (strain 1156)  
Thermoactinomyces vulgaris (strain 1150)  
Candida albicans (3153)  
C. parapsilosis (3104)  

Antigens were reconstituted at optimal dilutions determined by titration against control antisera. A. fumigatus was used at 24 and 0.6 mg/ml, other fungi and yeasts at 10 and 0.1 mg/ml, M. faeni and T. vulgaris at 6 and 0.6 mg/ml. A positive control (usually A. fumigatus) was included with each slide or simultaneous batch of 4 slides.

Sera

Some sera stored at −20°C up to several months were examined retrospectively. The majority of sera used in the study were examined prospectively from specimens submitted to the laboratory. No selection was exercised on the types of test done. Sera were examined simultaneously by double diffusion and CIE by the appropriate test antigens so that subsequent comparison could be made of the results obtained. Numbers of sera and the specific tests undertaken are shown in table 1.

These were grown for 3 days in glucose peptone broth at 26°C with stirring of the medium. The mycelial debris was filtered off, disintegrated in a Dyno Mill (Glen Creston), concentrated by dialysis against polyethylene glycol, and freeze dried.

were grown in a continuous culture apparatus and then processed as above. Some studies were made with Hollister-Stier C. albicans antigen used at a dilution of 1:10 or 1:40.

Table 1. Comparison of CIE and DD results: Retrospective study

<table>
<thead>
<tr>
<th>Test Antigen</th>
<th>No. of sera tested</th>
<th>No. negative both tests</th>
<th>No. positive both tests</th>
<th>No. positive DD only</th>
<th>No. positive CIE only</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus fumigatus</td>
<td>75</td>
<td>4</td>
<td>58</td>
<td>8*</td>
<td>5</td>
</tr>
<tr>
<td>Mieropolyspora faeni</td>
<td>10</td>
<td>0</td>
<td>9</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Thermoactinomyces vulgaris</td>
<td>10</td>
<td>6</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>36</td>
<td>5</td>
<td>17</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>C. parapsilosis</td>
<td>36</td>
<td>23</td>
<td>1</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Totals</td>
<td>167</td>
<td>38</td>
<td>86</td>
<td>12</td>
<td>31</td>
</tr>
</tbody>
</table>

*4 of these were negative on re-testing

Double diffusion

In the Ouchterlony test routinely used at the MRL (Murray & Buckley, 1969), 3 ml of borate-EDTA buffered agar is pipetted into 5 cm plastic petri dishes. While still molten, a jig is introduced to create a pattern consisting of a central well 6 mm in diameter, four peripheral wells 6 mm in diameter and 2 peripheral wells 2 mm in diameter. Edge to edge distances of the central and peripheral wells are 5 mm. After removal of the jig, test serum (60 µl) is placed in the centre well. Peripheral wells contain control antisera and two different test antigens. The sizes of the antigen wells are such that a tenfold difference in quantity of test antigen is obtained, corresponding in effect to about a tenfold difference in concentration. Plates are developed in humid cabinets at room temperature for 3 days, then washed for 3 days. Portions of the gels containing the well pattern are cut out, mounted on microscopic slides, dried and stained with Coomassie Brilliant Blue.
Table 2. Comparison of CIE and DD results: Prospective study

<table>
<thead>
<tr>
<th>Test Antigen</th>
<th>No. of sera tested</th>
<th>No. negative both tests</th>
<th>No. positive both tests</th>
<th>No. positive DD only</th>
<th>No. positive CIE only</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. fumigatus</td>
<td>306</td>
<td>200</td>
<td>30</td>
<td>4</td>
<td>72</td>
</tr>
<tr>
<td>A. flavus</td>
<td>14</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>A. nidulans</td>
<td>15</td>
<td>9</td>
<td>0</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>A. niger</td>
<td>14</td>
<td>10</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>A. terreus</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Micropolyspora faeni</td>
<td>54</td>
<td>42</td>
<td>8</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Thermoactinomyces vulgaris</td>
<td>54</td>
<td>49</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>44</td>
<td>29</td>
<td>2</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>C. parapsilosis</td>
<td>44</td>
<td>38</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Histoplasma capsulatum</td>
<td>11</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Allescheria boydii</td>
<td>10</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Totals</td>
<td>570</td>
<td>399</td>
<td>45</td>
<td>11</td>
<td>115</td>
</tr>
</tbody>
</table>

Results

Numbers of sera examined and numbers containing antibodies detectable by double diffusion (DD) and CIE respectively are shown in table 1 and 2.

The primary goal of the study was a comparison of CIE and DD procedures. Most sera submitted to the laboratory for antibody determinations are not accompanied by sufficient clinical information to allow any valid evaluations to be made for either procedure and their diagnostic value. In the few (41) instances where a diagnosis of aspergillosis was unequivocal or probable the correlation between presence of antibody and symptoms was appreciably better with CIE than with double diffusion. Of 30 sera from patients with symptoms consistent with a diagnosis of allergic aspergillosis, 11 were positive in both DD and CIE tests. An additional 14 were positive by CIE alone. In no instance was a serum positive by DD and negative by CIE. A separate study of the correlation between CIE results and the clinical aspects of aspergillosis will be reported elsewhere.

Interpretation

It was found that some experience was necessary in recognising positive CIE tests. Presence of two or more lines (fig. 2a) made readings simple. Difficulties in interpretation were not experienced when the lines were straight (fig. 2b) or curved away from the serum well (fig. 2c). On some occasions a positive reaction is indicated by the presence of precipitin spurs, where the antigen-antibody reaction occurs partly in the serum well (2d). A common finding was the occurrence of a single stained line between the two test wells located 1–2 mm from the serum well and closely following its outline (fig. 2e). Since this line was usually found in only one of the two pairs of test wells, it was originally thought to represent a specific line of precipitate. However, it was also occasionally seen in test plates containing serum but no antigen: furthermore lines of immune precipitate are characteristically straight rather than curved (2b), and often have cathode-directed

Fig. 2. Types of lines produced in CIE (see text for explanation).
deflections at their ends (2e). Inhibition of the equivocal lines (2e, f) by addition of antigen to the serum wells before electrophoresis showed that in some instances the lines were precipitins. Since such inhibition tests are impracticable as a routine, it was decided that all equivocal lines should be recorded as negative. All slides were read independently by both investigators, and the results compared. Attention was focussed on individual tests where readings differed, and agreement was reached on the criteria for positive and negative tests. All tests were read again. The overall coincidence of interpretations on the second reading was 94% - a level that is regarded as acceptable. The choice of concentrations for the specific antigens was based on comparative studies of a range of dilutions. For Aspergillus fumigatus, it was found that no additional reactions were observed at intermediate concentrations between 24 mg/ml and 0.6 mg/ml, and these were therefore eliminated. Reactions were seen at one or both concentrations used, and against one or both antigens prepared from strains no. 2109 and 2140.

It was noted that if only one antigen of A. fumigatus had been used for both DD and CIE tests, a substantial number of sera containing antibodies would have been overlooked. It is not yet known if the use of additional concentrations of antigen would result in the recognition of a sufficiently high number of positively reacting sera to justify the additional effort involved. Aspergillus antigens used routinely in the MRL differ from those used in many other laboratories in being derived from young (3 day) mycelia rather than culture filtrates. During the developmental phase of this work, it was found that the results obtained with MRL and commercially available reagents (Remarc, Pasteur Institute) showed no significant differences. All comparisons herein reported refer only to CIE and the Ouchterlony double diffusion used as a routine in this laboratory.

Discussion

The ultimate value of any serological test depends on the degree of correlation which exists with the diseased state. Both the test and its reading have to be reproducible. Introduction of a new test is unjustified unless it offers advantages over existing procedures. It is clear that CIE has two exploitable advantages when compared with the routine double diffusion test undertaken in this laboratory, viz. greater speed and sensitivity. Preliminary results can be obtained in 90 minutes, and on several occasions this has been of great value as a screening procedure. Greater sensitivity of a procedure is not necessarily an advantage, particularly when antibodies to the test antigen are common (e.g. Candida). The situation with antibodies to Aspergillus differs in the sense that they are not only uncommon in healthy subjects, but can be implicated in the disease process (Pepys, 1969). From the results obtained, it is clear that CIE allows the detection of many more sera with antibodies to A. fumigatus than is obtained with DD. It is normal practice in this laboratory to concentrate and re-test serum for antibodies to A. fumigatus when there are strong clinical grounds for the diagnosis of allergic aspergillosis. In a laboratory where several thousand sera are being screened annually, routine concentration of serum is wasteful of time and money, and in our experience largely unjustified by the results obtained. What is required in the serological evaluation of sera from patients with a possible diagnosis of allergic aspergillosis is a more sensitive test than double diffusion. Counterimmunoelectrophoresis has a demonstrable superiority in this sense and it is now being used routinely in this laboratory for detection of antibodies to A. fumigatus. In our hands, its greater sensitivity does not have the same advantage in tests for antibodies to other aspergilli, to Candida, or to the agents responsible for extrinsic allergic alveolitis. It is thought likely that in some instances optimal conditions have not yet been established for these systems. Evaluation of antigens that differ qualitatively and quantitatively from those used to date is in progress, and may lead to the adoption of CIE tests for antigens other than A. fumigatus.

Candida antibodies may be produced to cell wall (mannan) or cytoplasmic (protein) antigens. At present, the relative values of these two contrasting types of antigen in serological tests are not known. Mannan-free antigen of C. albicans has rarely been used in any published report, although affinity chromatography with sepharose-concanavalin A has been successfully used in its preparation (Ellsworth & Reiss, 1974; Longbottom & Brighton et al., 1974). Using CIE to screen patients for precipitins to C. albicans Dee and Rytel (1975) showed that antibodies were present in all 9 patients with candidiasis, in 63% of

### Table 3. Reactivities of 163 sera to one or both antigenic extracts of A. fumigatus used in CIE.

<table>
<thead>
<tr>
<th>Reacting to one or both concentrations of both antigens</th>
<th>DD</th>
<th>CIE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>66</td>
<td>91</td>
</tr>
<tr>
<td>Reacting to one or both concentrations of one antigen only</td>
<td>17</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>83</td>
<td>163</td>
</tr>
</tbody>
</table>

Totals 83 (50.9%) 163
patients with evidence of colonization rather than deep-seated Candida infection, and in 47% of patients with bacteraemia. The antigen used (Hollister-Stier) contains appreciable levels of mannan, and we believe that this would account for the apparent lack of specificity of the test. Mannan at high concentrations does not react in immunodiffusion tests but, within defined limits, an increasing proportion of sera become positive by DD and CIE as the concentration of mannan is reduced. We have noted that purified mannan reacts in CIE tests at very low concentrations: in one series of 100 sera, the majority (62%) reacted to 5 g/ml of mannan (unpublished results). Until further knowledge is gained on the diagnostic implications of serological reactivity to mannan and protein antigens respectively, the comparative value of CIE and DD remains uncertain. Such evaluations are currently in progress (Merz, Evans et al., 1975).

A significant finding was that many sera stored at \(-20\,^\circ\text{C}\), particularly those with low levels of precipitins, become non reactive by both CIE and DD. Loss of serum reactivity to \textit{C. albicans} in DD has already been reported by Taschdjian, Kozinn et al. (1972). In this context the difference in results between the retrospective and prospective studies is striking. Numbers of positive CIE tests revealed in the retrospective study were appreciably less than in the prospective study despite the fact that a much greater proportion of the stored sera had contained antibodies on initial testing. In addition, some of the stored positive sera were negative by DD when re-tested.

From the experience and results obtained to date, there is clear evidence that CIA has a role in the serodiagnosis of mycotic disease. It can be anticipated that further refinements and developments will define more clearly the circumstances where exploitable diagnostic correlations exist between clinical and laboratory findings.

**Summary**

Counterimmunoelectrophoresis (CIE) has been compared with agar gel double diffusion (DD) as a routine procedure for the serological examination of sera for antibodies to pathogenic and allergenic fungi and actinomycetes. The method was applied to a retrospective study of 167 sera stored at \(-20\,^\circ\text{C}\) and as a routine procedure in parallel with DD on 306 sera submitted to the Laboratory for mycoserological screening.

Mycelial antigens used at 2 dilutions reacted in CIE more frequently than in DD. Results with stored sera showed that CIE was more effective in detecting antibodies than DD, even when the serum no longer reacted on re-testing with DD. In the prospective study antibodies were detected by CIE but not by DD to \textit{Histoplasma capsulatum} (5 sera), \textit{Allescheria boydii} (7 sera) and \textit{Candida parapsilosis} (6 sera). Results with \textit{A. fumigatus} showed that 102 of 306 sera (33%) were positive by CIE compared with 34 (13%) by DD. Preliminary results showed a greater correlation between positive CIE results and allergic aspergillosis (25 of 30 sera), compared with only 11 of 30 by DD. The greater sensitivity of CIE suggests that it can be used in preference to concentration and re-testing of sera with low levels of antibody.

**References**


