

Detection of *Aspergillus* antigen and anti-*Aspergillus* precipitating antibodies by a new method: the radial immunoelectro-osmophoresis (RIEOP)

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Summary

A new method of Immunoprecipitation-in-gel, the Radial Immunoelectro-osmophoresis (RIEOP) has been applied to detect precipitating anti-*Aspergillus fumigatus* antibodies and to study the antigens present in various antigenic extracts of this fungus.

The results obtained with this method have been compared with those obtained when applying the Comparative Double Diffusion (CDD) and the Immunoelectro-osmophoresis (IEOP).

The data presented prove that the RIEOP method is more sensitive than CDD and the IEOP in: (1) detecting precipitating anti-*Aspergillus* antibodies, since (a) precipitating bands are detected with higher serum dilutions, and (b) when using undiluted serum, the number of precipitating bands obtained by RIEOP is higher; and (2) studying antigens present in the antigenic extracts, since (a) the number of antigens detected by RIEOP is higher than that by CDD, and (b) identity/non-identity reactions can be studied among the antigens present in the various extracts.

Introduction

The Ouchterlony Comparative Double Diffusion (CDD) and Immunoelectro-osmophoresis (IEOP) have been extensively used for the detection of precipitating antibodies against *Aspergillus* antigens in the sera of patients with pulmonary pathology induced by this fungus (Pepys & Turner-Warwick, 1975; Longbottom & Pepys, 1975). Identity/non-identity reactions among antigens present in different antigenic extracts can be studied by means of CDD (Ouchterlony, 1958), but this method is not very sensitive. Immunoelectro-osmophoresis is a more accurate method but does not permit the study of identity/non-identity reactions and, consequently, the possibility of false results is increased (Smith, 1976).

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A new method of Immunoprecipitation-in-gel has been developed in our laboratory, the Radial Immunoelectro-osmophoresis or RIEOP (Llopis, Sanchez-Cuenca & Gonzalez-Molina, 1979), which combines the advantages of the above methods, CDD and IEOP.

The intention of this work was to apply RIEOP to detect precipitating antibodies against *Aspergillus fumigatus*, as well as to examine the antigens present in different extracts.

Material and methods

Sera from nine individuals, eight males and one female, aged between 19–76, with pulmonary mycotic conditions were examined. The diagnosis was established according to the usual clinical, radiological and mycological criteria, and in all the cases, precipitating antibodies against *A. fumigatus* had been detected.

All the patients had undergone surgical operations to remove the mycetoma. In five cases, blood samples for these studies were taken at the time of diagnosis; in the remainder (Nos. 3, 4, 6 and 8) the blood sample was obtained 1–3 years after the surgical operation.

Sera from nine healthy individuals (aged 21–41) were used as controls. All the sera were negative for C-reactive protein. They were stored at -20°C until used.

Precipitating antibodies against *Aspergillus* antigens were investigated in all the unknown and control sera, both undiluted and diluted in saline solution by means of CDD, IEOP and RIEOP methods.

Commercial extracts of *A. fumigatus* from Hollister-Stier (Lot 811401) and Bencard (Batch 36/34/8) were used.

Immunoprecipitation methods

CDD method. As supporting medium 0.1% Agarose (Behringwerke), in barbiturate-sodium barbiturate buffer 0.05 M, pH 8.6, was used. After solidifying a 2.0 mm thick coat of gel, a central well and six peripheral wells of 4.0 mm were made. The distance between the wells (centre to centre) was 7.0 mm.

For the study of antigens, the peripheral wells were filled with the different antigenic extracts and the central one with the serum No. 1, which in a previous evaluation had shown a high titration of precipitating antibodies.

For the detection of precipitating antibodies, the peripheral wells were filled with serial dilutions of sera ranging from undiluted to 1:64 in saline, and the central well with one of the antigenic extracts (Hollister-Stier).

Incubation was carried out in a humidity chamber at 4°C for 48 hr and readings were made immediately after.

IEOP method. The supporting medium and buffer from Austragen (Landerlan) were used. The diluted/undiluted serum samples were placed in the cathodic side wells and the antigenic extract (Hollister-Stier) in the opposite wells. The electrophoresis time was 45 min by applying a constant voltage with an initial current of 60 mA. Readings were taken immediately after the electrophoresis had finished.

RIEOP method. The electrophoresis apparatus has been described elsewhere (Llopis *et al.*, 1979). It is composed of an upper and a lower vessel, between which the supporting medium is placed (Fig. 1).

The lower vessel is a plastic cylinder, open at the top. Its bottom contains a circular electrode and a cylindrical crown of absorbant material to support a circular disc of

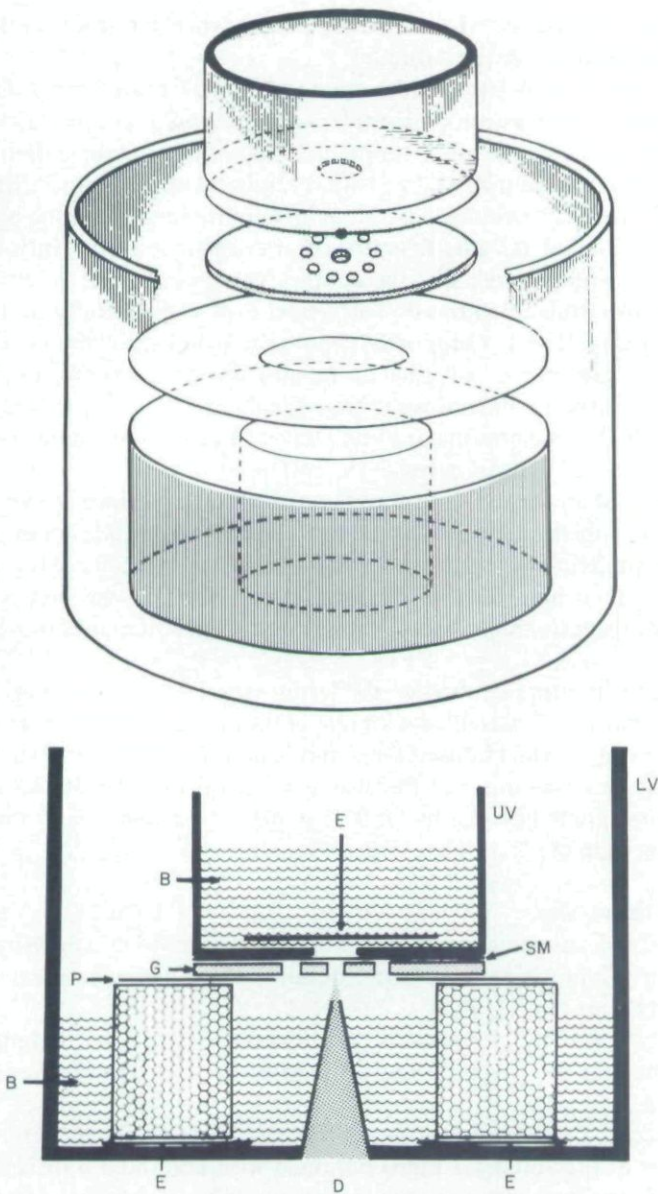


Fig. 1. Diagram of the electrophoretic apparatus used in RIEOP. Scheme and frontal section: LV, lower vessel; P, Whatman paper; D, drainage system; UV, upper vessel; SM, semi-permeable membrane; G, gel; B, buffer; E, electrodes.

Whatman paper. This vessel is filled with buffer to 1.0 cm from the top surface of the absorbant cylinder.

In the centre of this vessel there is a drainage system for the central well of the gel formed by a hollow cone open at the top, that is filled with absorbant cotton (D in Fig. 1). The top must reach the base of the gel. Its function is to drain the central well when

the endosmotic flux is directed to it, which occurs when the lower vessel houses the anode and the upper vessel the cathode.

The upper vessel is a plastic cylinder open at the top. It has a 5-mm hole in its centre. This hole is covered with a semi-permeable membrane adherent to the outside surface of the vessel. Over this membrane a 2-mm thick coat of 1.0% agarose (Behringwerke) in buffer is solidified and a central well and nine peripheral wells (4 mm each) are cut in the gel. The central well must coincide with the hole in the vessel, and the nine peripheral wells should be situated at 7 mm from the central well (centre-to-centre). After filling the central and peripheral wells with the samples, the upper vessel is inverted, filled with buffer and the electrode introduced. This vessel is placed centrally on the Whatman paper in such a way, that it is supported eventually by the absorbant cylindric crown.

To study the presence of antigens in the antigenic extracts and the identity/non-identity reactions, the peripheral wells were filled with the different extracts and the central well with the precipitating antibody serum. In this experiment the upper vessel housed the anode and the lower vessel the cathode.

A drainage system for the central well was unnecessary since the endosmotic flux was directed towards the periphery of the gel. The electrophoretic conditions were 3 hr at 3 mA (constant intensity) using veronal-veronal Na buffer 0.05 M pH 8. The readings were taken immediately after the electrophoretic run was completed. As controls, the antigenic extracts were tested against normal human serum (no antibody) and saline.

To detect precipitating antibodies, the serum samples were placed in the peripheral wells and the central well was filled with one of the antigenic extracts (Hollister-Stier). In this test, the upper vessel housed the cathode and the lower vessel the anode. Since the endosmotic flux was directed towards the central well the drainage system was necessary. A phosphate Na-Na₂ buffer 0.05 M, pH 5.5 was used and the conditions for electrophoresis were the same as above.

Evaluation of the results:

- (1) In the study of antigenic extracts, the following data were considered:
 - (a) number of precipitating bands obtained by testing extracts with specific antibody (serum No. 1), and
 - (b) Identity/non-identity reactions of antigens contained in different extracts.
- (2) In the detection of precipitating antibodies in sera, the following data were considered:
 - (a) presence or absence of precipitating bands when undiluted sera were used;
 - (b) number of precipitating bands obtained with undiluted sera; and
 - (c) the highest serum dilution giving at least one precipitating band (serum titre).

Results

Study of the antigens present in the antigenic extracts

CDD method. Four precipitating bands were obtained with Hollister-Stier extract, four with Bencard No. 2, and three with Bencard No. 1. Identity reactions among the antigens of the three extracts were found. There was no evidence of precipitating bands when the extracts were tested with sera controls and saline.

RIEOP method. Seven precipitating bands were obtained with Hollister-Stier extract, seven with Bencard No. 2 and six with Bencard No. 1 (Fig. 2). On comparing

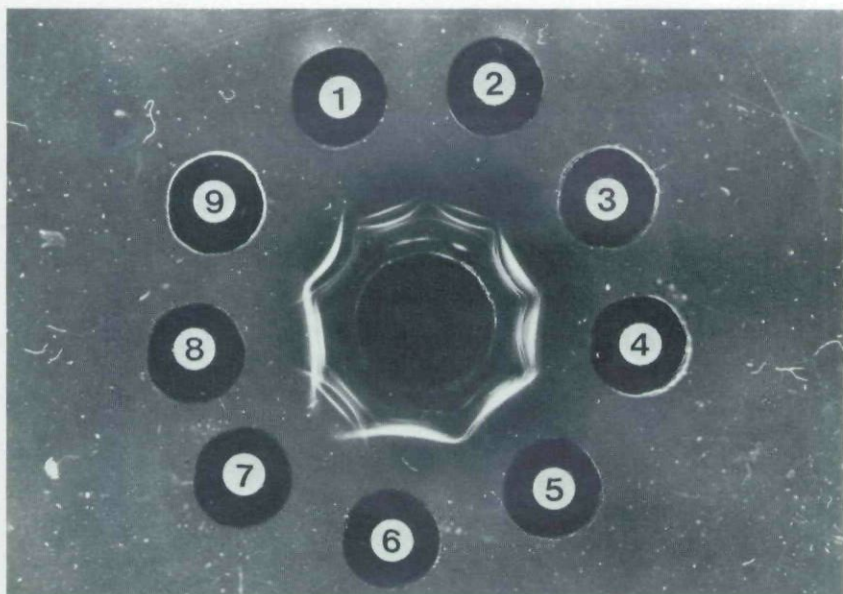


Fig. 2. Precipitating bands obtained by RIEOP, in the study of *Aspergillus fumigatus* antigens. The central well contains serum from Patient No. 1. Peripheral wells were filled with: 1, 2, 7, *Aspergillus* antigen Bencard No. 1; 3, 4, 9, *Aspergillus* antigen Bencard No. 2; and 5, 6, 8, *Aspergillus* antigen Hollister-Stier. Identity/non-identity reactions can be observed among antigens present in all the extracts. A variable number of precipitating bands is observed with each one of the extracts used (see text).

the Hollister-Stier extract (Well No. 8 in Fig. 2) with Bencard No. 1 (Well No. 9) complete antigenic identity was observed. A precipitating band in Well No. 9 gave a non-identity pattern reaction with those of Well No. 8, but gave an identity reaction with a band of an antigen present in Well No. 1.

Two precipitating bands nearest to Well Nos. 8 and 9 (Fig. 2) that display a complete identity reaction between themselves, show a non-identity reaction with the bands belonging to Bencard No. 1 (Well Nos. 1 and 7). In Fig. 2, the reliability of this method is demonstrated because when depositing the same extract in two adjoining wells (Bencard No. 1 in Well Nos. 1 and 2; Bencard No. 2 in Well Nos. 3 and 4; Hollister-Stier in Well Nos. 5 and 6) the identity between all the precipitating bands is complete. In no instance did the extracts show precipitating bands when confronted with control sera and saline.

Study of precipitating antibodies

CDD method. One to four precipitating bands were found in seven sera. The serum titration varied from undiluted to 1:4 (Table 1).

IEOP method. All the sera showed one to four precipitating bands, with a titration from undiluted to 1:16.

RIEOP method. All the sera showed one to six precipitating bands, with a titration from 1:2 to 1:32. As shown in Fig. 3, the precipitating bands show reactions of identity.

Table 1. Results obtained by CDD, IEOP and RIEOP in the detection of anti-*Aspergillus* antibodies in the serum of nine patients suffering from pulmonary aspergilloma. Central well contains *Aspergillus* extract from Hollister-Stier. Peripheral wells contain the unknown sera. Peripheral wells contain the unknown sera.

Patient No.	CDD		IEOP		RIEOP	
	ST*	NB†	ST	NB	ST	NB
1	1/4	4	1/16	4	1/32	7
2	1/4	2	1/16	2	1/32	3
3	neg.	—	1/2	1	1/2	1
4	neg.	—	1/1	1	1/2	1
5	1/2	2	1/8	3	1/8	3
6	1/2	2	1/4	3	1/8	3
7	1/1	1	1/2	1	1/4	1
8	1/1	1	1/2	1	1/2	2
9	1/1	1	1/2	1	1/4	2

* Serum titration (ST) is the highest dilution of serum giving at least one precipitating band.

† No. of bands (NB) indicates the maximum number of precipitating bands obtained with undiluted serum.

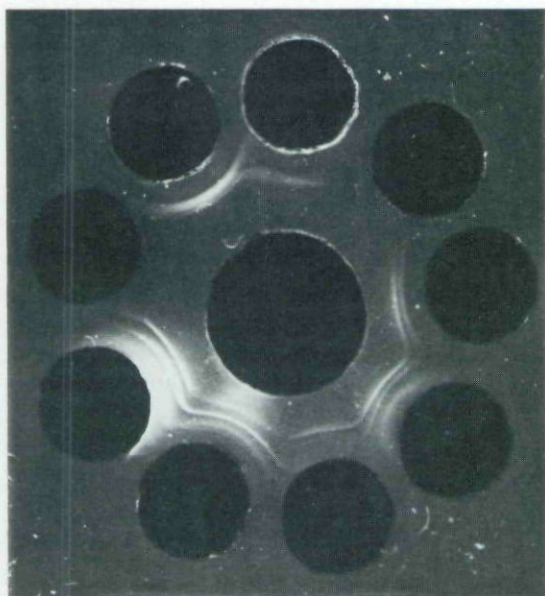


Fig. 3. Precipitating bands obtained by RIEOP in sera of nine patients suffering from pulmonary aspergilloma. Central well contains *Aspergillus* extract from Hollister-Stier. Peripheral wells contain the unknown sera. A variable number of precipitating bands is observed with the different sera, showing in some cases a complete identity reaction.

Discussion

Anti-*Aspergillus* precipitating antibodies have been found in most of the individuals suffering from a pulmonary disorder caused by *Aspergillus*. These antibodies are present in 60% of patients with allergic bronchopulmonary aspergillosis, when undiluted serum is used, and the frequency increases when the serum is concentrated two- or three-fold (Hoehne, Reed & Dickie, 1973). In the cases of pulmonary aspergilloma, precipitins are found in about 90% of the patients, and precipitation bands are more numerous.

When an appropriate antigen is used, circulating antibodies are found even in sera from healthy individuals (Bardana *et al.*, 1972). The finding of these antibodies is not diagnostic, but the presence of high titrations of precipitating antibodies could corroborate the clinical and mycological diagnosis. The detection of these antibodies depends on the type of extract used and the sensitivity of the method (Gerber & Jones, 1973).

Of the commonly used methods, the sensitivity of CDD is weak and the results, slow to obtain. For this reason, the IEOP method has been recommended as it is quicker (Hansen & Penny, 1974). However, identity/non-identity reactions cannot be determined by this method, and the risk of false positive results is increased.

In an attempt to combine the advantages of both CDD and IEOP we have designed the Radial Immunoelectro-osmophoresis. This method permits the application of an electric field to the gel, preserving the traditional form used in CDD. Thus, simple diffusion is replaced by an electrophoretic displacement resulting in quicker diffusion of the antigen and the antibody. By applying RIEOP, antigens of anodic charge are detected in a time and with a degree of sensitivity similar to those of IEOP. Furthermore, it permits the study of identity/non-identity reactions (Llopis *et al.*, 1979). Likewise, as we demonstrated (unpubl. data), RIEOP permits a more sensitive detection of precipitating antibodies against HBsAg, IgA, IgG and IgM, than CDD or IEOP.

When RIEOP is used to study *Aspergillus* antigens the number of precipitating bands is greater than that obtained by CDD and appears to be four to eight times more sensitive than CDD. Thus, of the seven positive sera by CDD, the RIEOP was positive with a serum titration eight times higher in four, four times higher in four and two times higher in one. Two negative sera by CDD were positive by RIEOP with a serum dilution of 1:2.

When comparing the results obtained by IEOP and RIEOP it is observed that RIEOP was twice as sensitive as IEOP, in six out of the nine sera, and equal in the remainder.

The number of precipitating bands obtained with some of our sera is lower than that described in the literature on pulmonary aspergilloma cases. We believe that it may be due to the time between surgery and the taking of a blood sample to investigate precipitating antibodies (more than a year in Case Nos. 3, 4, 6 and 8) as well as to the low potency of the commercial extracts employed.

In conclusion, the new RIEOP method is more sensitive and less time-consuming than traditional precipitation methods, and can therefore be used advantageously in the detection of precipitating antibodies. Furthermore, it can also be applied to the study of *Aspergillus* allergens and consequently, to the study and standardization of other antigens.

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