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STANDARDIZATION OF THE IMMUNOELECTROPHORESIS TEST WITH WHOLE AND PURIFIED HYDATID CYST FLUID ANTIGENS FOR THE DIAGNOSIS OF HUMAN HYDATIDOSIS

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SUMMARY

Optimal technical conditions were established for the performance of the immunoelectrophoresis (IEP) test for the diagnosis of human hydatidosis with whole (WHF) and purified (PHF) hydatid cyst fluid antigens. Electrophoresis for 90 minutes in 0.9% agarose, with a potential difference of 20 volts between the ends of the slide, was found to be adequate for the separation of both antigens. The use of rectangular-shaped antigen wells resulted in the detection of more precipitation bands with WHF but circular antigen wells were also suitable for PHF. The optimal antigen concentration for detection of diagnostic arc 5 with WHF was 200 mg dry weight per ml while 30 mg protein per ml were optimal for the identification of bands A and B with PHF. Standardization of these conditions makes it possible to evaluate simultaneously the comparative sensitivity and specificity of both antigenic preparations in the diagnosis of human hydatidosis by the IEP test.

INTRODUCTION

The value of the immunoelectrophoresis (IEP) test for the diagnosis of human hydatid disease has been studied by several investigators [1, 2, 5, 7–10] using whole hydatid cyst fluid (WHF) [1] or purified hydatid cyst fluid antigens (PHF) [4]. No false positive reactors were observed in non-hydatid sera, when the diagnosis was based exclusively on the detection of *Echinococcus granulosus*-specific arc 5 [1] with WHF, or the presence of bands A and/or B with PHF [7].

The sensitivity of the former procedure, however, is apparently higher than that achieved with PHF although it is difficult to make direct comparisons since the techniques described in the literature differ in many important aspects which could be expected to influence the results. For example agarose [1, 2, 5, 8–10] and agar [4, 7] have been employed as supporting media; antigen well shape and size have been variable and antigens of different concentrations and host source have been used [1, 2, 4, 5, 7–10]. Furthermore, identical serum samples have not been examined in a direct comparative way in order to obtain a valid evaluation of the sensitivity and specificity of these differing procedures. For this study we describe

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an attempt to standardize two of the most recently developed variants of the IEP test as a prerequisite to a definitive comparative examination of their diagnostic merit.

MATERIALS AND METHODS

Antigens

A 10-liter pool of hydatid cyst fluid was collected [6] from the lungs and livers of naturally infected sheep at slaughter. Immunoelectrophoretic characterization using sheep antiserum to ovine WHF [6] showed the presence of *E. granulosus* arc 5 together with 13 other parasite antigens. Two aliquots of this lyophilized antigen were reconstituted in sodium veronal buffer pH 8.2 to contain final concentrations of 50 and 200 mg dry weight/ml respectively, for use in the IEP tests.

The purified sheep hydatid fluid (PHF) antigen was prepared as described by ORIOL et al. [4]. Briefly, 10% concentrated whole hydatid cyst fluid was dialyzed against phosphate buffer, centrifuged and the supernatant dialyzed against acetate buffer. The precipitate formed was resuspended in the phosphate buffer and the globulins removed by precipitation with ammonium sulfate. This procedure was repeated and immunoelectrophoretic analysis of these hydatid fluid fractions against rabbit antisera to PHF revealed the presence of the A and B antigens [4] and two other minor components. IEP of the PHF against rabbit antisera to normal sheep lungs revealed one host component. No bands were detected against normal sheep liver. The PHF antigen was used in IEP at concentrations of 5, 10, 20, 30, 40 or 50 mg protein per ml.

Immunoelectrophoresis (IEP) tests

Our comparative evaluation involved varying one at a time the conditions of antigen concentration, time of electrophoresis, supporting media and antigen well shape and size, attempting to maintain all other parameters constant in each set of observations.

WHF and PHF preparations were each examined critically in IEP tests using both 0.9% agarose and 1% agar as described by CAPRON et al. [2] and WILLIAMS et al. [7], respectively, to determine if the morphology of arc 5 and bands A and B would be recognized equally well in these two supporting media. Glass slides (75 × 25 mm) were used [2, 8]. Homologous hyperimmune rabbit sera and sera from two preoperative hydatidosis patients, one of which was only weakly reactive, were employed. Standard antigen concentrations of 200 mg dry weight per ml for WHF [2] and 10 mg protein per ml for PHF [7] were used (Figs. 1 and 2). Antigen wells were rectangular (4 × 1 mm) and circular-shaped (1.5 mm diameter) [2, 7] and the antigens were electrophoresed for 90 minutes at a potential difference of 20 volts.

The effect of the size and shape of the antigen well on the morphology of the diagnostic precipitation bands was also studied (Fig. 3). The antigen wells evaluated were rectangular (4 × 1 mm) and circular-shaped (1.5 and 3 mm diameters) [2, 7]. The WHF concentration was again 200 mg dry weight per ml and that of the PHF was 10 mg protein per ml [1, 7]. Agarose (0.9%) was employed as the supporting medium of the reactions on the basis of the above results. Sera and other IEP test conditions were performed as described above.

The IEP test was also performed using the WHF antigen at concentrations of 50 and 200 mg dry weight per ml [1, 7] (Fig. 4) and the PHF at concentrations of 5, 10, 20, 30, 40 and 50 mg protein per ml (Fig. 5) to determine the effect of the antigen concentration on the characteristic morphology of arc 5 and bands A and B. Sera and other IEP test conditions were as described above.

The optimal time of the run was studied using the WHF (200 mg dry weight per ml) and PHF (30 mg protein per ml) antigens. Electrophoresis was carried out for 45, 60, 90, 120 and 150 minutes at a potential difference of 20 V between the two ends of the slides (Fig. 6). The run was monitored by the addition of from-phenol blue [3] on the gel below the antigen well. Sera and other IEP test conditions were as described above.

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Immunoelectrophoresis test for the diagnosis of hydatid disease using 1% agar and 0.9% agarose as supporting media.

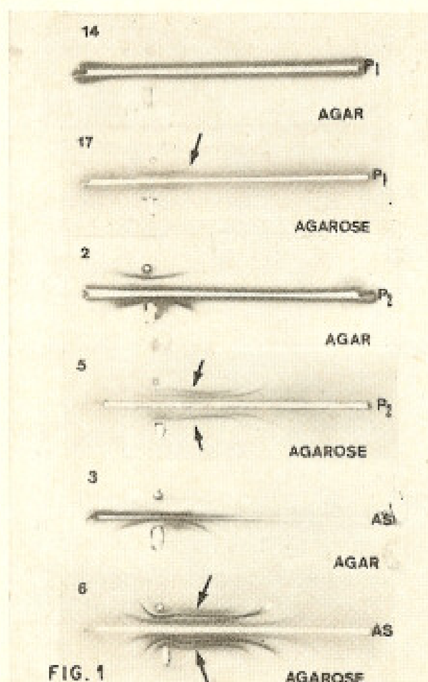

Fig. 1 — Antigen, whole sheep hydatid fluid (WHF) at a concentration of 200 mg dry weight per ml. P_1 and P_2, sera from two hydatidosis patients, including a weak serological reactor (P_1); *AS*, rabbit antisera to WHF. Arrows indicate the presence of arc 5.

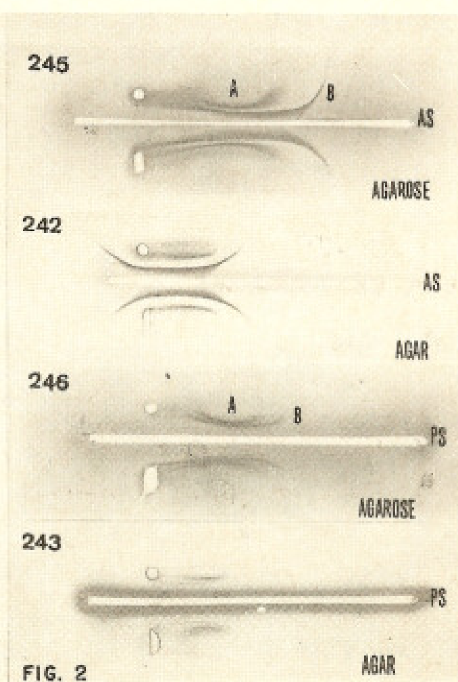

Fig. 2 — Antigen employed was purified sheep hydatid fluid (PHF) at a concentration of 10 mg protein per ml. *AS*, homologous rabbit antisera; *PS*, serum of a hydatidosis patient. *A* and *B* indicate bands A and B, respectively.

RESULTS

Agarose (0.9%) was found to be superior to agar (1%) in the IEP test for hydatidosis using both WHF and PHF antigens (Figs. 1 and 2). The characteristic appearance of the *E. granulosus*-specific arc 5 developed only in agarose, both with homologous hyperimmune rabbit serum and preoperative sera from hydatidosis patients (Fig. 1). The improved resolution provided by agarose was particularly evident with serum from a weakly reacting hydatidosis patient (P_1, Fig. 1). Bands A and B in PHF were formed in both supporting media but were more clear and distinct in 0.9% agarose (Fig. 2).

The characteristic appearance of bands A and B was not influenced by the size and shape of the antigen wells (Fig. 3). These diagnostic arcs were readily recognized when either rectangular (4 × 1 mm) or circular-shaped antigen wells (1.5 and 3 mm diameter) were used. With WHF, however, the appearance of arc 5 was optimal and more bands (other than arc 5) were detected when the antigen was electrophoresed in rectangular (eleven bands) rather than in circular wells (9 bands).

Fig. 4 shows that the expression of arc 5 was more distinct and the resolution improved when WHF was used at a concentration of 200 rather than 50 mg dry weight per ml, particularly when rectangular wells were used.

Figure 5 illustrates the effect of PHF concentration on the appearance of bands A and

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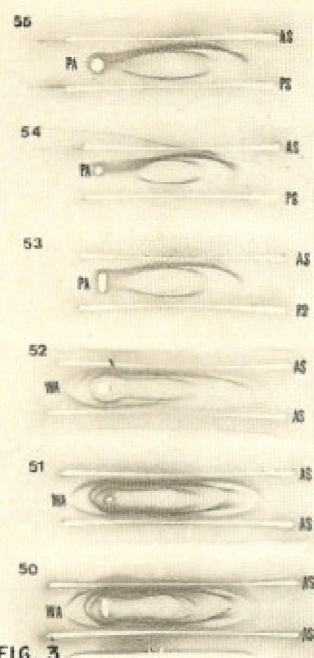

Fig. 3 — Effect of circular (3 and 1.5 mm diameter) and rectangular-shaped (4 x 1 mm) antigen wells. Whole (WA) and purified (PA) hydatid fluid antigens at concentrations of 200 mg dry weight per ml and 10 mg protein per ml respectively. *AS*, homologous rabbit antisera; *PS*, serum from a hydatidosis patient.

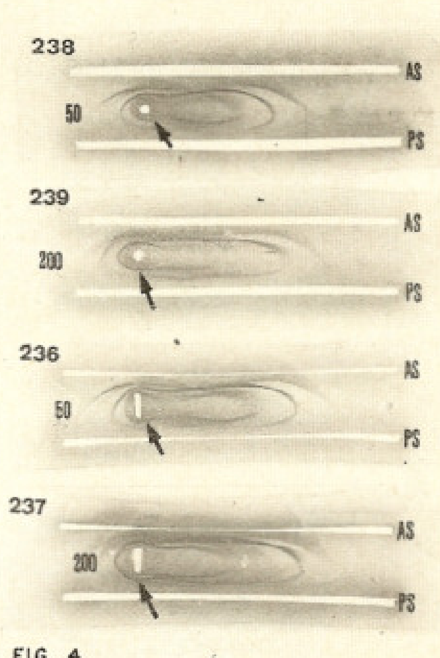

Fig. 4 — Effect of whole sheep hydatid fluid concentrations of 50 and 200 mg dry weight per ml. *AS*, homologous rabbit antisera. *PS*, serum from a hydatidosis patient. Circular (3 mm diameter) and rectangular (4 x 1 mm)-shaped antigen wells. Arrows indicate the presence of arc 5.

B. These were more clearly developed at concentrations of 30, 40 and 50 mg protein/ml. In order to conserve antigen 30 mg protein/ml were used thereafter.

When electrophoresis of WHF and PHF was carried out for 45, 60, 90, 120 and 150 minutes, it was found that optimal separation and resolution of arc 5 and bands A and B was achieved with 90 minutes (Fig. 6). At this time the bromphenol blue dye had migrated 35 mm in the anodic region.

DISCUSSION

The present study has demonstrated the superiority of 0.9% agarose [1] over 1% agar [4, 7] as a supporting medium for the IEP test for hydatid disease using either whole [1] or purified [4, 7] hydatid fluid antigens. Arc 5 and bands A and B [4, 7] were readily identifiable whether rectangular [1] or circular-shaped antigen wells [4] were employed. The use of rectangular wells seemed to result in the appearance of a greater number of bands and a more distinct morphology of arc 5 in WHF. This point could find practical application in the study of post-operatory sera by the IEP test for prognostic purposes. The number of bands is reported to wane gradually in the absence of other hydatid cyst in operated patients [1, 2, 8].

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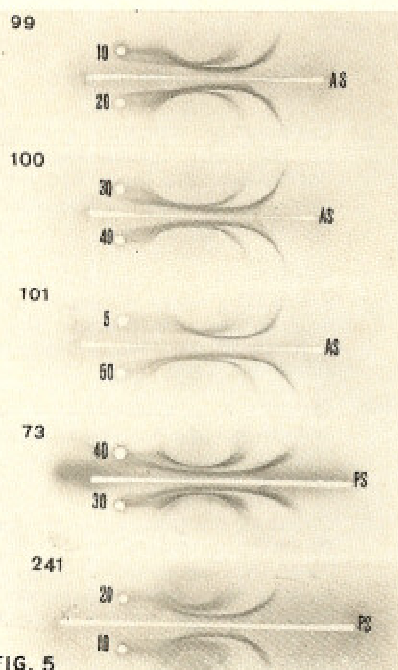

Fig. 5 — Effect of purified hydatid sheep fluid antigen concentrations of 5, 10, 30, 40 and 50 mg protein per ml. *AS*, homologous rabbit antisera; *PS*, serum from a hydatidosis patient.

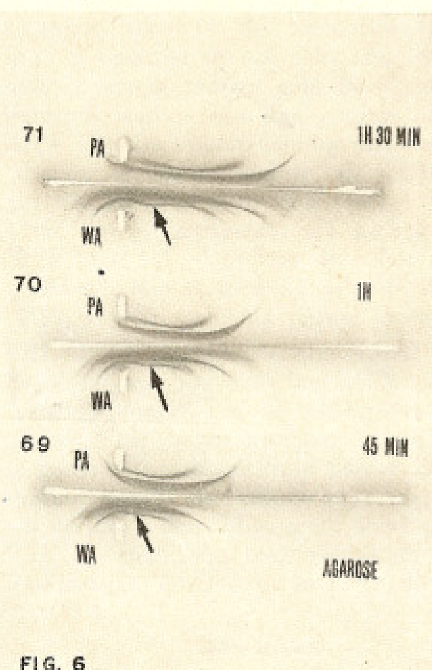

Fig. 6 — Effect of the time of electrophoresis of the antigen on the morphology of the arc 5 (indicated by arrow) and the A and B bands. *PA* and *WA* were purified and whole sheep hydatid fluid antigens at concentrations of 30 mg protein per ml and 200 mg dry weight per ml, respectively.

An association was also observed between the time of electrophoresis of the antigen and the morphology of the bands (Fig. 6). The optimal time for electrophoresis was 90 minutes at which time the appearance of arc 5 was most characteristic. Since the spot of bromphenol blue placed below the antigen well had migrated 35 mm under these conditions, the adequate mobility of the antigen may also be standardized in terms of the distance migrated by the dye as suggested by CHORDI & KAGAN [3]. This reduction of the time of electrophoresis from the 2 hours 30 minutes used previously [1, 2, 5, 8, 9] increases the efficiency of the test by allowing the examination of more serum samples. It is now routinely used in our laboratories with excellent results.

The optimal antigen concentrations employed to detect arc 5 and bands A and B in sera from hydatid patients and homologous rabbit antisera were 200 mg dry weight per ml for the WHF and 30 mg protein per ml for the PHF. The use of lower antigen concentrations however, influenced the quality of the diagnostic arcs formed by these same sera, suggesting that the diagnostic sensitivity of the IEP test may be reduced at lower antigen concentrations. This might be especially true if weakly reactive sera were used. These considerations may account, at least in part, for the lower sensitivity observed [7] with WHF and PHF at concentrations of 50 mg dry weight per ml and 10 mg protein per ml, respectively, than when 200 mg dry weight per ml of WHF have been used [1, 2, 5, 8-10].

In summary, electrophoresis of both WHF and PHF antigens in 0.9% agarose at a potential difference of 20 volts across a 75 × 25 mm slide for 90 minutes or until the bromphenol blue marker moves 35 mm from the antigen well was optimal for both antigens. The optimal concentration of WHF was 200 mg dry weight per ml as used by CAPRON et al. [2] and that for PHF 30 mg protein per ml. The rectangular (4 × 1 mm) antigen well [2] is preferable for the WHF although circular (1.5 or 3 mm diameter) wells [7] were equally adequate for the PHF.

Since the criteria of positivity for the IEP test using WHF and PHF antigens are based on the recognition of arc 5 and bands A and/or B with patient's serum, the technical variables studied were found to be crucial to the interpretation of the test. These findings permit a basis for a valid comparison on the relative sensitivity and specificity of IEP tests involving these two antigen preparations.

RESUMEN

Estandarización de la prueba de inmunoelectroforesis con antígenos de líquido hidatídico total y purificado para el diagnóstico de la hidatidosis humana.

Los Autores estudiaron las condiciones óptimas de ejecución de la prueba de inmunoelectroforesis (IEF) para el diagnóstico de la hidatidosis humana empleando antígenos de líquido hidatídico total y purificado. Pudo establecerse como adecuada para la separación de ambos antígenos una electroforesis de 90 minutos de duración en agarosa al 0.9%, con 20 v de diferencia de potencial. El uso de hoyos rectangulares dio lugar a la formación de más bandas de precipitación con el antígeno total; para el antígeno purificado, los hoyos circulares fueron igualmente adecuados.

La concentración de antígeno más conveniente para el reconocimiento del arco 5 de valor diagnóstico — fue 200 mg peso seco/ml, mientras que 30 mg de proteína/ml resultaron óptimas para la identificación de las bandas A y B con el antígeno purificado. La estandarización de estas condiciones permitiría la evaluación simultánea de la sensibilidad y especificidad comparativas de ambas preparaciones antigénicas en el diagnóstico de la hidatidosis humana mediante la prueba de IEF.

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