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Purification and partial immunochemical characterization of a low molecular mass, diagnostic *Echinococcus granulosus* immunogen for sheep hydatidosis

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Abstract: A hydatid specific antigen of 8 kDa molecular mass was affinity-purified from crude hydatid cyst fluid. Some of the epitopes recognised by antibodies in the sera from sheep with hydatidosis were periodate-sensitive. The purified 8 kDa antigen was observed to be a thermo-stable glycoprotein in its immunochemical characteristics. By immunofluorescence on acetone-fixed protoscolices anti-8 kDa monospecific IgG antibodies indicated the existence of the 8 kDa molecule on the hooklets of protoscolices. The purified antigen was used in an enzyme-linked immunosorbent assay for the detection of specific antibodies in sera from sheep hydatidosis. Eighteen (90%) of 20 sera from sheep hydatidosis had antibodies to purified 8 kDa antigen while none of the sera from other parasitic infections or uninfected animals had any detectable levels of antibodies to 8 kDa antigen. Thus, the data on localization and recognition of hydatid specific 8 kDa molecule suggested that this may be one of the major molecules for specific immunodiagnosis and for modulating the hydatid disease process in infected hosts.

Key words: Echinococcus granulosus; Glycoprotein; Monospecific antibodies; Enzyme-linked immunosorbent assay; Sheep hydatidosis

Introduction

Hydatidosis or unilocular echinococcosis is caused by the larval stage of the cestode *Echinococcus granulosus. E. granulosus* is worldwide in its distribution and constitutes an important economic and public health problem [1,2]. *E. granulosus* develops successively in definitive (dog) and intermediate (cattle, sheep and man) hosts, and the consequences of this cyclozoonosis are of both veterinary and medical importance [1–3]. Sheep are the principal intermediate hosts of *E. granulosus* in most endemic regions of the world [2–4]. Casoni's intradermal test and a variety of serological tests have been used for diagnosis of hydatid infection in sheep, including repre-

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sentative tests from each of the categories described for human diagnosis [1–6]. Enzyme-linked immunosorbent assay (ELISA) procedures using a variety of antigens (protoscolices, hydatid cyst fluid, cyst wall or antigen B/5) have been applied for the immunodiagnosis of hydatidosis [1–3,7]. However, the occurrence of non-responders in infected sheep, as well as problems of cross-reactivity with *Taenia hydatigena* and *T. ovis* infections, limits their usefulness for immunodiagnosis of hydatidosis in sheep [1,3,8,9]. Thus, there is a dire need to identify the hydatid specific antigen(s). Recently, we [10], as well as Maddison et al. [11], reported that 8 kDa hydatid cyst fluid (HCF) antigen was specific for hydatidosis in immunoblot assay and never recognised by any sera from other helminthic infections or healthy controls, suggesting that the 8 kDa antigen is specific for hydatidosis. Therefore, isolation and immunochemical characterization of such antigen is essential to understand the immunological responses in the host : parasite relationship in order to develop a simple immunodiagnostic tool. Thus, the present study was carried out to isolate and investigate the partial immunochemical nature of affinity purified hydatid-specific 8 kDa antigen. In addition, the recognition of 8 kDa antigen by sera from sheep with hydatidosis was also investigated.

Materials and Methods

Antigen

Fertile hydatid cysts from sheep were obtained from a local abattoir. The HCF was aspirated aseptically, centrifuged, dialysed extensively against distilled water, lyophilised and stored at −20°C as described earlier [10]. The protein content of HCF was estimated [12].

Anti-hydatid hyperimmune serum

Anti-hydatid hyperimmune serum (AH-HIS) was raised in rabbit as described earlier [13].

Affinity purification of 8 kDa antigen

Hydatid-specific antigen with a molecular mass of 8 kDa was identified by us, employing Western immunoblot assay [10]. Antibodies to 8 kDa antigen were raised in rabbit as described earlier [14]. The specificity of antibodies to 8 kDa antigen eluted after SDS-PAGE was confirmed previously in immunoblots of crude hydatid cyst fluid, by obtaining a single band at corresponding to 8 kDa [13]. The IgG fraction of the hyperimmune serum to 8 kDa antigen was purified by DEAE-cellulose chromatograpy [15], dialysed, concentrated by lyophilization and labelled as anti-8 kDa IgG antibodies. The protein content of anti-8 kDa IgG antibodies was estimated [12]. The 8 kDa hydatid-specific antigen was affinity purified [15]. Pooled fractions of 8 kDa antigen were desalted through Sephadex G-25 (Pharmacia Fine Chemical AB, Uppasala, Sweden) and the eluates were concentrated by lyophilization (Kontron Instruments, Zurich, Switzerland). The protein contents of lyophilised materials were estimated [12].

Monospecific antibodies to affinity purified 8 kDa Antigen

Antiserum to affinity purified 8 kDa antigen was raised in rabbit by immunizing with 50 ug of affinity purified protein per dose. The schedule for immunisation has been described [13]. The anti-8 kDa monospecific IgG fraction was obtained by DEAE-cellulose chromatography [15].

Protein characterization

Electrophoresis and immunoblotting The purity and specificity of affinity purified antigen was investigated by resolution on SDS-PAGE under reducing conditions with a 15% separating gel [16] followed by immunoblotting [17]. The molecular mass standards used were: Carbonic anhydrase (29 kDa), Ovalbumin (45 kDa) and bovine serum albumin (66 kDa). The molecular mass of hydatid cyst fluid antigens recognised was estimated from a logrithmic plot of the migration of standards.

The 8 kDa molecule was also analyzed by two-dimensional electrophoresis, initially by isoelectric focusing in tube gels containing 4% ampholytes in the pH range of 4–6 and 1% ampholytes in the pH range of 3–10 (Mini-PROTEAN II 2-D Cell, Bio-Rad) and then by SDS-PAGE in 15% separating gels [18]. Gels were

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stained or transblotted to nitrocellulose membranes. In addition to two-dimensional electrophoresis, the 8 kDa molecule, was also analyzed on crossed immunoelectrophoresis in 1.2% low electroendosmotic agarose (Sigma Chemical Co., USA) [15].

Biochemical characterization of affinity purified protein The nature of affinity purified 8 kDa antigen was characterised immunochemically in terms of alterations in its immunoreactivity after heat treatment, proteolytic digestion and periodate oxidation [19]. Lipid digestion was done with lipase (Sigma) as described by Rolfe and Finegold [20].

Localisation of hydatid specific 8 kDa antigen The indirect immunofluorescent antibody test (IFAT) was employed to assess the localization of the 8 kDa molecule in protoscolices of *E. granulosus* [15]. Briefly, freshly aspirated HCF from sheep was centrifuged at 2000 × *g* for 30 min at 4°C. The pellet was washed extensively with PBS (pH 7.2). The acetone-fixed protoscolices were

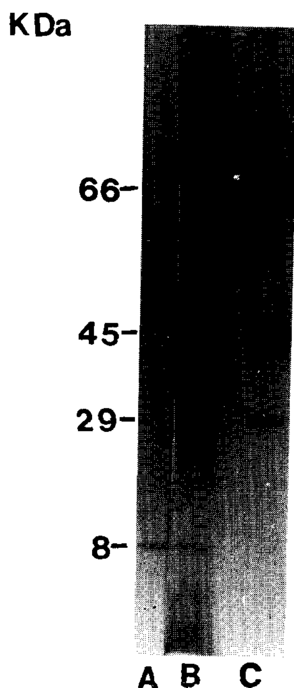

Fig. 1. SDS-PAGE of affinity purified 8 kDa antigen (lane A) and crude hydatid cyst fluid antigen (lane B). Molecular mass markers (lane C).

Fig. 2. Crossed immunoelectrophoresis for analysis of crude hydatid cyst fluid with AH-HIS (**A**) and affinity purified 8 kDa molecule with AH-HIS (**B**).

incubated with anti-8 kDa monospecific IgG antibodies at room temperature for 30 min. The washed smears were treated with 50 μl of 1:25 diluted anti-rabbit (immunoglobulin G) fluorescein isothiocynate (FITC) conjugate in 1:10000 Evans blue for 30 min at room temperature. The smears, mounted in glycerol/buffered saline (pH 6.8) were examined under the fluorescent microscope (Nikon, Japan).

Sera

Serum samples from slaughtered sheep and pigs with hydatidosis and cysticercosis were collected from a local abattoir. At autopsy the subcutaneous tissues, skeletal musculature, viscera, peritoneum and diaphragm were critically examined for cysts (hydatid or cysticercus).

Hydatidosis (n = 20) The microscopic examination of hydatid cyst fluid revealed the presence of protoscolices of *E. granulosus*. The sera of these sheep had anti-hydatid antibodies as assessed by micro ELISA employing crude hydatid cyst fluid as antigen [10].

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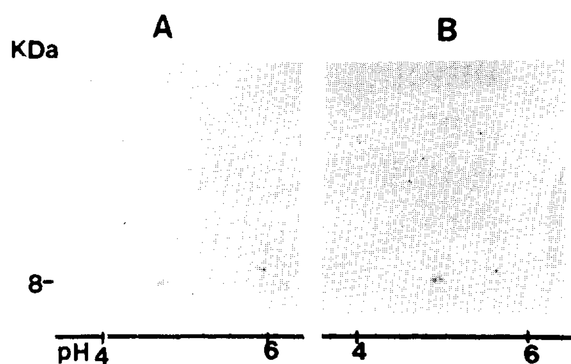

Fig. 3. Analysis of affinity purified 8 kDa antigen reacted with anti-8 kDa molecule by two-dimensional gel electrophoresis (**A**) and immunoblotting of purified 8 kDA antigen reacted with anti-8 kDA monospecific antibodies (**B**).

Cysticercosis (n = 15) Serum was collected from sheep ($n = 7$) and pigs ($n = 8$) having monospecific infection of either *T. hydatigena, T. ovis, T. solium* or a mixture of gastrointestinal nematodes. These sera had anti-cysticercus antibodies [10].

Normal Controls (n = 32) These animals (sheep/pigs) had no cysts after post-mortem examination. Their sera had no antibodies to hydatid cyst fluid or to cysticercus antigens.

Recognition of 8 kDa molecule by animal sera

A total of 67 serum samples from sheep and pigs were tested for the detection of anti-8 kDa antibodies by ELISA.

The ELISA was performed basically as described by Voller et al. [21]. In brief, the ELISA was performed by coating each well with 1 μg antigen in 0.05 M carbonate buffer (pH 9.6). The wells were blocked with 3% BSA and incubated with sera from sheep or pigs. After treatment with anti-sheep HRP conjugate or anti-pig HRP conjugate (Jackson Immune Research Lab, Baltimore, USA) the colour reaction was developed with 6 N H_2SO_4 and optical density was measured at 492 nm in an ELISA reader (Eurogenetics, Belgium).

Statistical analysis

Data are expressed as mean $\pm$ standard deviation (SD). Results were compared by Student's *t*-test.

Results

Isolation and physiochemical properties of affinity purified molecule

The hydatid-specific 8 kDa molecule was successfully eluted from specific antibodies coupled to an affinity column. Purity of the affinity purified 8 kDa molecule was assessed: a single band of 8 kDa was obtained following resolution on SDS-PAGE under reducing and denaturing conditions (Fig. 1, lane A). A single immunoprecipitin arc with the 8 kDa antigen was also seen in crossed immunoelectrophoresis with AH-HIS (Fig. 2). Further the purity of affinity purified 8 kDa molecule was assessed by two-dimensional

Table 1

Effect of heat, pronase, lipase and sodium metaperiodate on immunoreactivity of pooled sera from sheep hydatidosis as assessed by ELISA

Treatment	Mean [a] OD after exposure of antigen to heat, pronase, lipase or sodium metaperiodate
Heat at	
25°C	0.631 ± 0.051
60°C	0.585 ± 0.038
100°C	0.570 ± 0.047
Pronase	
0 $\mu g\ ml^{-1}$	0.641 ± 0.071
10 $\mu g\ ml^{-1}$	0.151 ± 0.051 *
100 $\mu g\ ml^{-1}$	0.130 ± 0.090 *
1 000 $\mu g\ ml^{-1}$	0.115 ± 0.070 *
Lipase	
0 $\mu g\ ml^{-1}$	0.550 ± 0.052
50 $\mu g\ ml^{-1}$	0.495 ± 0.091
100 $\mu g\ ml^{-1}$	0.555 ± 0.101
1 000 $\mu g\ ml^{-1}$	0.510 ± 0.094
Sodium metaperiodate	
0 M	0.612 ± 0.041
0.025 M	0.441 ± 0.058
0.05 M	0.371 ± 0.091 *
0.1 M	0.297 ± 0.090 *
Pronase followed by sodium metaperiodate	
0 M + 0 $\mu g\ ml^{-1}$	
[1000 $\mu g\ ml^{-1}$	0.631 ± 0.075
+	
0.1 *M*]	0.051 ± 0.094 *

[a] Each value is the mean of two sets tested in triplicate.

* $P < 0.01$ compared with untreated antigen.

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electrophoresis and immunoblotting: a single spot was observed (Fig. 3 A, B). The monospecificity of anti-8 kDa antibodies was revealed by immunoblotting: a single band at 8 kDa in crude hydatid antigen (Fig. 4, lane B) or affinity purified 8 kDa antigen (Fig. 4, lane C) was observed. Purified 8 kDa antigen was also recognized by pooled sera from sheep hydatidosis (Fig. 4, lane D).

Biochemical characterization of purified protein

Heat treatment, even at 100°C for 10 min, caused no significant ($P > 0.01$) alteration to its immunoreactivity (Table 1). Proteolytic digestion with pronase reduced the immunoreactivity progressively at concentrations of 10, 100 and 1000 μg/ml. Similarly, sodium metaperiodate oxidation also reduced the immunoreactivity significantly ($P < 0.01$). However, the immunoreactivity was not affected by lipase treatment (Table 1).

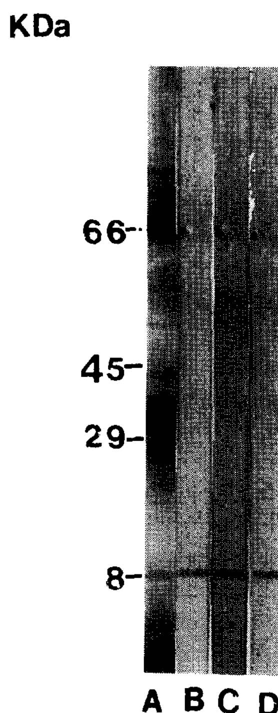

Fig. 4. Western immunoblots of HCF reaeted with AH-HIS (lane A) and monospecific antibodies to affinity purified 8 kDa antigen (lane B); Western immunoblots of purified 8 kDa antigen reacted with anti-8 kDa monospecific antibodies (lane C) and pooled sera from sheep infected with hydatid (lane D).

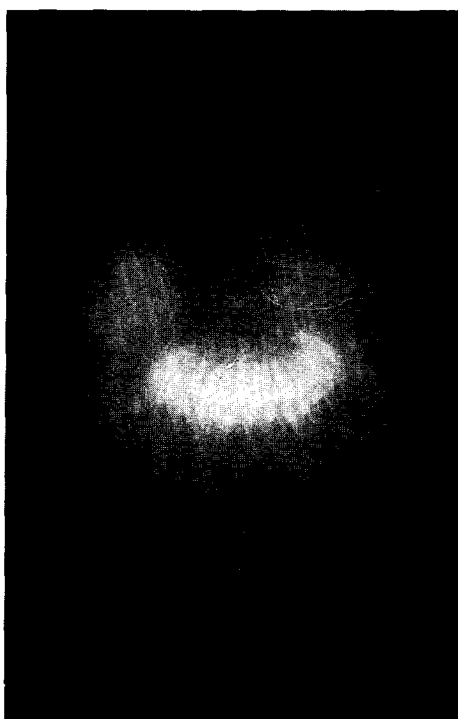

Fig. 5. Localization of affinity purified 8 kDa antigen by employing anti-8 kDa monospecific IgG antibodies as a fluorescent probe.

Localisation of hydatid-specific 8 kDa antigen

Employing anti-8 kDa monospecific IgG antibodies as a fluorescent probe, 8 kDa antigen was found to be localised on the hooklets of the protoscolices of *E. granulosus* (Fig. 5).

Recognition of 8 kDa antigen by sheep hydatid sera

Employing the 8 kDa molecule as antigen in ELISA, the mean OD with sera from uninfected sheep was 0.21 ($\pm$0.09). A serum with OD value of not less than 0.39 (mean + 2 SD of OD value of sera from uninfected animals) was considered to contain anti-8 kDa-specific antibodies. Eighteen (90%) of 20 sera from sheep hydatidosis had specific antibodies to the 8 kDa antigen while none of the sera from other parasitic infections or

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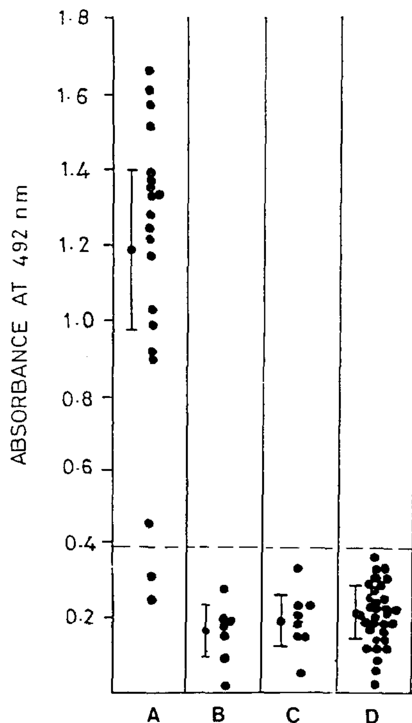

Fig. 6. Recognition of 8 kDa purified molecule of *E. granulosus* in micro-ELISA by animal serum from (**A**) sheep hydatidosis, (**B**) sheep cysticercosis, (**C**) pig cysticercosis and (**D**) uninfected sera. The broken lines represent the cut-off OD value.

uninfected sheep or pigs had specific antibodies to the 8 kDa antigen (Fig. 6).

Discussion

In the present study, we were able to purify *E. granulosus*-specific 8 kDa antigen from hydatid cyst fluid. The purified 8 kDa molecule did not dissociate into subunits when exposed to both reducing and denaturing conditions in SDS-PAGE or two-dimensional gel electrophoresis; therefore, it is a single polypeptide. The immunoreactivity of the antigen was not altered when exposed to heat. However, the immunoreactivity of the antigen was reduced after treating it with pronase, this might be due to degradation of the protein epitopes of the antigen. The immunoreactivity of the antigen was significantly ($P < 0.01$) altered after treatment with sodium metaperiodate indicating the presence of carbohydrate epitope(s) in the purified 8 kDa antigen. It was, therefore, regarded as a thermo-stable monomeric glycoprotein. Since the reaction was not totally abolished by periodate oxidation, it is possible that the remaining epitopes are periodate-insensitive. This is partly supported by the inability of pronase, despite its low specificity, to completely destroy the antigenic determinants of the purified 8 kDa antigen. However, the combined treatment (pronase followed by metaperiodate) of the antigen abolished its immunoreactivity, indicating the glycoprotein nature of the purified antigen.

The biochemical properties of purified 8 kDa antigen were similar to those described for the subunits of antigen B. Like these subunits, purified 8 kDa antigen is not affected by reduction at boiling [1–3,22]. It has been shown recently that antigen B cross reacts with the sera from other helminthic infections [1,23]. Moreover, antigen B has been classified as a lipoprotein. In contrast to these properties, we observed that 8 kDa purified antigen was neither a lipoprotein nor did it cross-react with antibodies from other helminthic infections. Thus, it is felt that 8 kDa antigen and antigen B are different biologically as well as in their biochemical characteristics.

The ability of hydatid-specific 8 kDa antigen to detect antibodies in 90% of sera from sheep hydatidosis, combined with its specificity makes it a suitable antigen for serodiagnosis and seroepidemiological surveys. Such surveys are important for assessment of disease prevalence in sheep as well as for monitoring the efficacy of control measures once implemented.

Since the 8 kDa molecule was found to be hooklet-specific, its specific localization makes it a likely target for immunologic attack. These hooklets are organs for the attachment of the parasite to the target cells. We, therefore, tend to feel that the 8 kDa molecule on the hooklets of *E. granulosus* protoscoleces may be one of the major molecules modulating the hydatid disease process in the infected hosts.

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References

1 Schantz, P.M. and Gottstein, B. (1986) Echinococcosis (Hydatidosis). In: Immunodiagnosis of Parasitic Diseases. Vol. 1 (Walls, K.F. and Schantz, P.M., Eds.), pp. 69–107, Academic Press, Orlando, FL.

2 Rickard, M.D. and Lightowlers, M.W. (1986) Immunodiagnosis of hydatid disease. In: The Biology of *Echinococcus* and Hydatid Disease (Thompson, R.C. A., Ed.), pp. 217–240, George, Allen and Unwin, London.

3 Lowson, J.R. and Gemmell, M.A. (1983) Hydatidosis and cysticercosis: The dynamics of transmission. Adv. Parasitol 22, 261–308.

4 Bortoletti, G., Gabriele, F., Seu, V. and Palmas, C. (1990) Epidemiology of hydatid disease in Sardinia: A study of fertility of cysts in sheep. J. Helminthol. 64, 212–216.

5 Gemmell, M.A. and Lowson, J.R. (1986) Epidemiology and control of hydatid disease. In: *Echinococcus* and Hydatid Disease (Thompson, R.C.A., Ed.), pp. 197–216. George, Allen and Unwin, London.

6 Kagan, I.G. (1968) A review of serological tests for the diagnosis of hydatid disease. Bull. WHO 39, 25–37.

7 Tassi, C., Dottorini, S., Scalise, G. and Geranio, N. (1981) *Echinococcus granulosus*: Diagnosis of human hydatid disease by the indirect fluid haemagglutination reaction with antigen from hydatid fluid and scoleces. Int. J. Parasitol. 11, 85–89.

8 Yong, W.K. and Heath, D.D. (1979) 'Arc 5' antibodies in sera of sheep infected with *Echinococcus granulosus, Taenia hydatigena* and *T. ovis*. Parasite Immunol. 1, 27–38.

9 Richard, M.D. (1979) The immunological diagnosis of hydatid disease. Aust. Vet. J. 55, 99–104.

10 Kanwar, J.R., Kaushik, S.P., Sawhney, I.M.S., Kamboj, M.S. Mehta, S.K. and Vinayak, V.K. (1992) Specific antibodies in sera of patients with hydatidosis recognised by immunoblotting, J. Med. Microbiol. 36, 46–51.

11 Maddison, S.E., Slemenda, S.B., Schantz, P.M., Fried, J.A., Wilson, M. and Tsang, V.C.W. (1989) A specific diagnostic antigen of *Echinococcus granulosus* with an apparent molecular weight of 8 kDa. Am. J. Trop. Med. Hyg. 40, 377–383.

12 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with folin phenol reagent. J. Biochem. 193, 265–275.

13 Kanwar, J.R. and Vinayak, V.K. (1992) The significance of free and immune-complexed hydatid specific antigen(s) as an immunodiagnostic tool for human hydatidosis. J. Med. Microbiol. 37, 396–403.

14 Vinayak, V.K., Singla, N., Dutt, P. and Mahajan, D. (1990) The 66 kDa polypeptide as ligand receptors molecule of promastigotes of *Leishmania donovani*. Jpn. J. Med. Sci. Biol. 44, 109–123.

15 Hudson, L. and Hay, F.C. (1980) Practical immunology, 2nd edn., pp. 33–100. Blackwell Scientific Publications, Oxford.

16 Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227, 680–685.

17 Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350–4254.

18 O'Farrel, P.H. (1975) High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250, 4007–4021.

19 Kum Kum, Khanna, R. and Vinayak, V.K. (1991) Characterization of surface associated antigens of axenic *Giardia lamblia* trophozoites and their recognition by human sera. Ind. J. Med. Res. 93, 40–46.

20 Rolfe, R.D. and Finegold, S.M. (1979) Purification and characterization of *Clostridium difficile* toxin. Infect. Immun. 25, 191–201.

21 Voller, A., Bidwell, D. E. and Barlett, A. (1976) Enzyme immunoassay in diagnostic medicine. Theory and Practice. Bull. WHO 53, 55–65.

22 Oriol, C. and Oriol, R. (1975) Physicochemical properties of a lipoprotein antigen of *Echinococcus granulosus*. Am. J. Trop. Med. Hyg. 24, 96–100.

23 Shepherd, J.C. and McManus, D.P. (1987) Specific and cross-reactive antigen of *Echinococcus granulosus* hydatid cyst fluid. Mol. Biochem. Parasitol. 25, 143–154.

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