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Echinococcus granulosus, Taenia hydatigena, T. ovis: Evaluation of cyst fluids as antigen for serodiagnosis of larval cestodes in sheep

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ABSTRACT

In order to monitor the progress of New Zealand's hydatids eradication campaign, a specific, serological, diagnostic test is required to identify infected sheep. An indirect haemagglutination test, using pyruvic aldehyde-stabilized sheep erythrocytes as the antigen carrier, was developed for the serodiagnosis of larval cestode infections of sheep. Using cyst fluid from *Echinococcus granulosus, Taenia hydatigena* and *T. ovis* as the antigens in this test, it was shown that the larval cestode species, responsible for an infection in sheep harbouring a single specific infection, could be identified by the higher titre given with the homologous antigen, in comparison to that given with the heterologous antigens. Sera of sheep infected with two or more species were also tested by this method, and the only specific infections to be diagnosed by differential titres were those due to the presence of live *E. granulosus* cysts. These antigens cannot be used for the diagnosis of specific larval cestode infections in the field because of the cross-reactivity between cyst fluids. However, the test did show that infection with larval cestodes could be diagnosed on a non-specific basis.

INTRODUCTION

In New Zealand, three larval cestode species are known to be present in sheep. These are *Echinococcus granulosus, Cysticercus tenuicollis* and *C. ovis,* all of which share the same sheep-dog-sheep life cycle.

Studies on the serology of larval cestode infections in sheep are few. Lamy *et al.*[11] studied complement fixation tests, whilst others[3][19] used an indirect haemagglutination (IHA) test. The latter appeared to give more consistent results in identifying existing infections in animals than did the former; probably because complement fixation tests have been shown to monitor infections with dead cysts better than infections with live cysts[7]. These observations agreed with findings from studies on serodiagnosis for human hydatidosis[10][21]. Lamy *et al.*[11] and Blundell-Hasell[3] reported that a positive reaction was always obtained when a larval cestode antigen was reacted with homologous antisera, but a certain amount of cross-reaction was also observed with heterologous sera. For instance, antigens of *T. hydatigena* cyst fluid were found to react with sera of sheep infected with *E. granulosus;* the reverse titration also gave significant cross-reaction[3][11][19]. No study has yet been made of any of the antigens from *T. ovis.* Nevertheless, Blundell-Hasell[3] reported that sera from sheep infected with *T. ovis* in the field gave a positive reaction in the IHA test using either *T. hydatigena* or *E. granulosus* cyst fluid as the antigens.

The IHA method has been used, with many modifications, by many workers for the immunodiagnosis of helminth parasite infections. The IHA test for hydatidosis has been performed using cells treated with tannic acid[6], glutaraldehyde[1][24], benzidine[4] and formol[2]. Varela-Diaz *et al.*[23] compared these variants of the IHA test for the immunodiagnosis of human hydatidosis. They considered the technique employing tannic acid as the choice IHA test, because of its higher sensitivity over the other 3 techniques. The reason for the loss of sensitivity by these 3 techniques could be due to the distortion of the erythrocyte after treatment with these chemicals and the subsequent modification of the attached proteins[13]. Conversely, others have claimed that glutaraldehyde as a coupling agent in the IHA test provided a more sensitive result in the diagnosis for bovine cysticercosis than tannic acid[5][20]. Pyruvic aldehyde has the advantage over other aldehydes in that cells treated with pyruvic aldehyde retain their characteristic biconcave shape, it does not cause lysis of the cells and there is no tendency of the treated cells to spontaneously agglutinate[13]. In the situation where we envisage the IHA test being used as a sero-epidemiological diagnostic tool, large numbers of sera will be tested. Pyruvic aldehyde treatment of cells has the advantage over the tannic acid technique in that it results in large quantities of stable and storable erythrocytes. Pyruvic aldehyde, and other aldehyde-stabilized erythrocytes sensitized with antigens, were reported to be stable for at least 3 months[1][2][12]. This IHA technique allows the screening of large numbers of sera using the same batch of reagents. One person can test 150 serum samples a day using the manual microtitration technique.

This paper reports a study devoted to the development of the IHA test using pyruvic aldehyde-stabilized sheep erythrocytes sensitized with cyst fluid from either *E. granulosus, T. hydatigena* or *T. ovis.* In the absence of specific diagnostic antigens for these three larval cestode species, we also investigated the possiblity that differential titres with the 3 antigens against a test serum might be useful for specific diagnosis.

MATERIALS AND METHODS

Antigens

Cysts of *E. granulosus* and *T. hydatigena* were collected from sheep killed at the local abattoir. The *E. granulosus* cyst fluid (EgCF) from both lung and liver cysts was pooled and centrifuged at 2000 g for 30 minutes at 4°C. The supernatant was washed with 5 volumes of distilled water through a PM10 ultrafiltration membrane+ at 4°C and then frozen at –20°C in 3 ml aliquots. The *T. hydatigena* cyst fluid (ThCF) was pooled and similarly processed.

To obtain *T. ovis* cyst fluid (ToCF), eight 6-month-old lambs were each orally infected with 8000 fresh *T. ovis* eggs. The animals were slaughtered 5-6 weeks after infection. All the muscle tissue of each carcass was sliced at about 8 mm intervals to locate the cysts and the clear fluid from each cyst was aspirated. The ToCF collected from each animal was pooled and treated as for EgCF.

The same batches of pooled antigens were used throughout this study. The protein concentration of each antigen pool was estimated by the method of Lowry *et al.*[14]. EgCF contained 35 mg protein/ml; ThCF 28 mg protein/ml; and ToCF 10 mg protein/ml.

Antisera

Experimentally infected sheep. Sheep were reared from birth on cestode-egg-free pastures and, at 3 months old, each animal

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+ Amicon Corporation, Lexington, Massachusetts, U.S.A.

was given either a single, double or triple infection of cestode eggs. Of the sheep given single oral infections, 19 received 2000 *E. granulosus* eggs, 14 received 2000 *T. hydatigena* eggs and 18 received 2000 *T. ovis* eggs. Serum samples were collected from these sheep at necropsy, which ranged from 1 to 12 months after infection for *E. granulosus* infected animals, and from 1 to 3 months for the *T. hydatigena* and *T. ovis* infected animals.

The sheep infected with eggs from two cestode species were fed 2000 eggs of *T. ovis* or 1000 eggs of *E. granulosus* or *T. hydatigena,* and were challenged orally 3 months later, either with the homologous species or one of the two heterologous species. These sheep were then necropsied 3 months after challenge and serum samples collected.

In the triple infections, 50 sheep were fed with a mixture of 1000 eggs of each of the 3 larval cestode species. The sheep were necropsied 3 months later and serum samples collected.

Naturally infected sheep. Forty-four 5-year-old ewes were obtained from a private farm, the pastures of which were known to have been contaminated with cestode eggs for at least 6 years. Sera were collected from the sheep at necropsy.

Indirect haemagglutination test

The indirect haemagglutination (IHA) test was performed using pyruvic-aldehyde-treated, sheep red blood cells as the antigen carriers. Red blood cells were prepared from venous blood collected in an equal volume of Alsever's solution[15] from uninfected larval-cestode-free sheep. A modification of the direct-protein-attachment procedure, described by Ling[13], was used to treat the cells with pyruvic aldehyde+ and sensitize them. The three antigens were used at a concentration of 3 mg protein/ml. This gave maximum titres with positive reference sera in a chessboard titration of antigens. The positive reference sera were obtained from sheep experimentally infected with either *E. granulosus, T. hydatigena* or *T. ovis,* and in which antibodies against the homologous cyst-fluid antigens had earlier been demonstrated by precipitation in agar plates. All sheep sera were heated in a water-bath, at 60°C for 1 hour before use, to prevent non-specific agglutination. Eagle's minimum essential medium*, containing 2% foetal calf serum, was used as diluent. Two-fold serial dilutions were used for all sera in disposable microtiter 'V' plates++, starting with the undiluted serum. Each test trial was read after the tray was incubated at room temperature for 3 hours and, again, after overnight storage at the same temperature. The readings were made according to Salk's method[18], that is, a layer of cells spread evenly across the bottom of the well was considered positive; a button of cells without ragged edges, negative. The titre of each serum was therefore the highest serum dilution that gave a positive reaction. Controls for each test trial included wells containing non-sensitized, pyruvic-aldehyde-treated cells and test serum; non-sensitized, pyruvic-aldehyde-treated cells and diluent; sensitized cells and reference positive serum; sensitized cells and reference negative serum from uninfected sheep; and sensitized cells and diluent. The same pool of pyruvic-aldehyde-treated cells was used throughout this study. This included the initial development work to standardize the test system, and the testing of all the sheep sera.

RESULTS

Sera from experimentally infected sheep (Table I)

Agglutination was observed when sera from sheep with single infections were reacted against the homologous, and heterologous, antigens; the greatest reactions being with the homologous antigens. These sera, collected from sheep with infections ranging from 1-12 months for *E. granulosus* and 1-3 months for both *T. hydatigena* and *T. ovis,* showed that age of infection did not influence the outcome of the test. A range of titres was obtained, but the titre against the homologous antigen was always the greatest, compared with those against the heterologous antigens. At necropsy the numbers of viable cysts found per animal for *E. granulosus* ranged from 50-200, for *T. hydatigena* 30-200, and for *T. ovis* 10-100. No statistically significant correlation was found between the serum titre and the number of cysts in each animal.

In the double and triple infection groups, all sheep at necropsy were found to harbour viable cysts of the species administered and their sera-agglutinated cells sensitized with each of the three antigens. Sera of sheep infected twice with the same species of larval cestode gave the highest titres against their homologous antigen. Sera of sheep infected twice with different species of larval cestode generally gave the highest titres against antigens from the infecting species. However, with sheep infected with both *T. hydatigena* and *T. ovis* 6 out of the 14 animals gave the greatest reactions with the heterologous antigen (EgCF).

In animals infected with two species of larval cestode, prior infection with *E. granulosus* tended to result in a higher titre for *E. granulosus* than for *T. hydatigena* or *T. ovis infections (Table I).* However, when sheep were infected with three different species simultaneously the over-shadowing of *E. granulosus* titres was less apparent.

Sera from naturally infected sheep (Table II).

All 44 ewes harboured *T. hydatigena* cysts. Twenty-four were also infected with *E. granulosus;* 13 of these had live cysts, ranging in number from 1 to 6 cysts per animal. Cysticerci of *T. ovis* were only sought in the viscera and on the surface of the carcasses. One *T. ovis* cyst was found in the heart of one animal.

The correlation analyses between the presence of cysts versus the ranking of titres showed that the presence of viable *E. granulosus* cysts is positively correlated with the highest titres against *E. granulosus* cyst fluid ($r = 0.71$, $P<0.001$), and negatively correlated with the titres against *T. hydatigena* cyst fluid ($r = -0.65$, $P<0.001$).

DISCUSSION

Serological cross-reaction between the three larval cestode species of *E. granulosus, T. hydatigena* and *T. ovis* found in sheep has been reported previously[3][11][16][19]. In those studies, positive reactions were observed in all sera, regardless of whether the antigen from one species was used to test the sera from sheep infected with the homologous or heterologous species. Similar observations were made in the present study. However, the degree of antigen-antibody reaction in the homologous system was greater than that in heterologous systems. Under identical optimal conditions of testing, the IHA titres from sera of sheep infected with one larval cestode species were at least 2 dilutions higher against the homologous cyst fluid antigen, compared with the titres of the same serum against the two heterologous antigens. In the absence of the availability of a species-specific diagnostic antigen for any of these parasites, the species responsible for an infection in singly infected sheep could be identified by this criterion of differential IHA titres.

Fairly similar results were obtained with sera from sheep experimentally infected with two larval cestode species – with the exception of sera from sheep infected with both *T. hyda-*

+ Aldrich Chemical Company, Wisconsin, U.S.A.
++ Cooke Microtiter System, Dynatech Laboratories Incorporated, Virginia, U.S.A.
* Grand Island Biological Company, New York, U.S.A.

tigena and *T. ovis*. Why *T. hydatigena* and *T. ovis* together given to sheep should induce higher cross-reacting anti-bodies in serum against antigens of EgCF is not clear, although a serological enhancement effect with a combination of other parasitic infections has been reported in sheep before[11]. On the other hand, animals infected twice with the same species showed some suppression of the cross-reacting antibodies in the serum; perhaps due to competition for antigenic binding sites in the host[17].

The degree of sensitivity of the IHA test reported in field trials for cysticercosis and hydatidosis in man, sheep, cattle and pig has been variable[3][6][8][9][16]. This variation could be explained by the criteria which each worker used to record positive results, and also by the type of antigens they used. However, when false positive reactions are not taken into account, the IHA test has been reported to be consistently highly sensitive[5][20]. In this study, a positive reaction was one that gave a titre against any of the three antigens used. Of the 44 sera collected from sheep with natural infections, all but 2 were positive to at least 1 of the antigens; giving a sensitivity of 95%. These titres were, in general, lower than those obtained with sera from experimentally infected animals. Similar observations with sera from experimentally, and naturally, *C. bovis*-infected cattle were made by Frick and Süsse[5] and Tailliez *et al.*[20]. This difference could be indicative of lighter infections experienced by animals in the field, in comparison to the heavy experimental infections.

Despite the apparent sensitivity of the IHA test for detecting cestode infected sheep, the results of this study showed that in the naturally infected ewes, the IHA test only identified specifically animals infected with live *E. granulosus* cysts. The IHA test has been claimed to be more sensitive in showing up infections with viable cysts than infections with dead cysts. Kagan *et al.*[10] reported that the IHA test, using hydatid cyst-fluid as the antigen, was very sensitive in the detection of positive clinical echinococcus infections in humans (96.7% of 30 sera). They noted that the IHA titres were very high and the agglutination of the sheep cells very strong in all these cases. Varela-Diaz *et al.*[22] found that with sera from human hydatid cases, diagnostically significant IHA titres correlated very well with the subsequent detection of *E. granulosus* arc 5 precipitation band by the immunoelectrophoresis test; the latter is the only technique available to date which gives immunological confirmation of the disease in humans, and the only technique with which no false positive results have been found. Geerts *et al.*[7], in their review of available serodiagnostic tests for bovine cysticercosis, also stated that the IHA test is most sensitive in diagnosing live mature *Cysticercus bovis* infections. The results from the present

TABLE I: INDIRECT-HAEMAGGLUTINATION (IHA) TEST OF SERA OF EXPERIMENTALLY INFECTED SHEEP WHEN *ECHINOCOCCUS GRANULOSUS* (EgCF), *TAENIA HYDATIGENA* (ThCF) AND *T. OVIS* (ToCF) CYST FLUIDS WERE USED AS THE ANTIGENS

	Treatment	Total number of sheep examined	Number of sheep giving their highest IHA titre against the antigens used		
			EgCF	ThCF	ToCF
A.	SINGLE INFECTION GROUP[1]				
	Echinococcus granulosus	19	19 (8.2)*	0 (4.2)	0 (5.4)
	Taenia hydatigena	14	0 (5.4)	14 (8.4)	0 (5.0
	Taenia ovis	18	0 (6.5)	0 (5.8)	18 (8.9)
B.	DOUBLE INFECTION GROUP[2]				
	E. granulosus – E. granulosus	7	7 (7.4)	0 (0.6)	0 (0.9)
	T. hydatigena – T. hydatigena	7	0 (6.9)	7 (9.7)	0 (2.9)
	T. ovis – T. ovis	7	0 (4.3)	0 (1.4)	7 (7.1)
	E. granulosus – T. hydatigena	8	7 (9.5)	1 (5.8)	0 (3.0)
	T. hydatigena – E. granulosus	7	3 (7.1)	4 (6.3)	0 (3.4)
	E. granulosus – T. ovis	6	6 (9.0)	0 (3.7)	0 (6.3)
	T. ovis – E. granulosus	7	1 (4.3)	0 (0.9)	6 (5.4)
	T. hydatigena – T. ovis	7	4 (9.4)	2 (7.7)	1 (7.4)
	T. ovis – T. hydatigena	7	2 (6.9)	1 (5.7)	4 (8.6)
C.	TRIPLE INFECTION GROUP[3]				
	E. granulosus + T. hydatigena + T. ovis	50	23 (8.0)	18 (8.4)	9 (5.8)
D.	UNINFECTED CONTROL GROUP	8	0 (0)	0 (0)	0 (0)

1. Each animal was given one oral dose of 2000 cestode eggs at 3 months of age.
2. Each animal was infected orally with 1000 eggs of *E. granulosus* or *T. hydatigena* or 2000 eggs of *T. ovis* at 3 months of age and then infected again with either the homologous species or one of the two heterologous species at 6 months of age.
3. Each animal was infected once orally with a mixture containing 1000 eggs each of the 3 larval cestode species at 3 months of age.

* Figures in brackets are the mean titres expressed as $\log_2$.

TABLE II: INDIRECT-HAEMAGGLUTINATION (IHA) TEST OF SERA OF 5-YEAR-OLD NATURALLY INFECTED SHEEP WHEN *ECHINOCOCCUS GRANULOSUS* (EgCF), *TAENIA HYDATIGENA* (ThCF) AND *T. OVIS* (ToCF) CYST FLUIDS WERE USED AS THE ANTIGENS

Echinococcus granulosus	Total number of sheep examined	Number of sheep giving their highest IHA titre against the antigens used		
		EgCF	ThCF	ToCF
Viable cysts	13	12 (6.6)*	1[1] (4.8)	0 (3.9)
Dead cysts	11[2]	2 (2.6)	7 (3.7)	0 (1.7)
No cyst	20[3]	2 (2.0)	17 (3.6)	0 (1.4)

1. This animal also had 1 *T. ovis* cyst in the heart.
2. Including 2 sheep where one gave no titre and the other gave the same low titre ($\log_2 3$) for the 3 antigens.
3. Including 1 sheep which gave no titre against the 3 antigens.

* Figures in brackets are the mean titres expressed as $\log_2$.

study also demonstrate this characteristic of the IHA test.

In conclusion, the IHA test, using the highest titre obtained against the cyst-fluid-antigens of *E. granulosus, T. hydatigena,* or *T. ovis* as the criterion of positive diagnosis, is not a suitable method for either serodiagnostic, or epidemiological, study in areas where more than one of these larval cestode species are present, because of the complex immunological cross-reactivity among these three cestodes. However, the test may be useful in detecting flocks with viable *E. granulosus* infections, and may also be useful for non-specific detection of infection with larval cestodes.

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