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Evaluation of chick red blood cells stabilized with various aldehydes in the indirect haemagglutination (IHA) test for diagnosis of neurocysticercosis

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Abstract

The present study was carried out to evaluate the use of chick cells in the indirect haemagglutination (IHA) test for the diagnosis of neurocysticercosis. Patients attending epilepsy clinic, Jawaharlal Institute of Postgraduate Medical Education and Research, (JIPMER) Hospital, Pondicherry were included in the present study. The study included 15 cases of neurocysticercosis with single small cystic lesion, 15 cases of neurocysticercosis with multiple cystic lesions, 15 cases of neurological disorders other than cysticercosis (meningitis, etc.) and 30 normal healthy people as controls. Results of the study showed that agglutinated chick red blood corpuscles (RBCs) settled after 30 to 45 min of incubation at room temperature and demonstrated a definite pattern of haemagglutination with the cysticercus positive sera. In case of most of the sera from patients with neurocysticercosis, the IHA using double aldehyde stabilized (DAS) chick cells gave 2-4 times higher titer than the chick cells stabilized by single aldehydes such as formaldehyde, glutaraldehyde or pyruvic aldehyde. The IHA employing DAS chick cells was found to be equally sensitive with that of the IHA using DAS human RBCs. A total of 27 out of 30 sera from the cases of neurocysticercosis were positive for cysticercus antibodies by the IHA employing the chick DAS as well as human DAS cells sensitized with cysticercus antigen. In conclusion, results of the present study show that the IHA using DAS chick RBCs instead of human 'O' RBCs will be a useful procedure with equal sensitivity for the diagnosis of neuerocysticercosis.

Keywords: Indirect haemagglutination, Chick RBCs, Double aldehyde stabilized cells, Haemagglutination, Neurocysticercosis.

Introduction

Indirect haemagglutination (IHA) test is a technically simple and inexpensive technique using antigen sensitized erythrocytes for demonstration of serum antibodies in a variety of parasitic diseases. The test has been proved to be useful for diagnosis of various parasitic diseases *viz.,* schistosomosis (Van Goel *et al.*, 2002), echinococcosis (Parija *et al.*, 1985; Rigano *et al.*, 2002), leishmaniosis (Iqbal *et al.*, 2002), malaria (Ray *et al.*, 1983), amebic liver abscesses (Parija *et al.*, 1989; Wiwanitkit, 2002), lymphatic filariosis and toxoplasmosis (Soliman *et al.*, 2001). Erythrocytes from different sources have been used by various workers in the IHA test. The frequently and widely used erythrocytes are from sheep. Other sources are from human of 'O' blood group (Herbert, 1978), turkey (Singh *et al.*, 1980), goose (Mannen *et al.*, 1984), bovine and swine (Holmgren, 1973). Avian cells are usually recommended because being nucleated, they are heavier, they settle quickly, so results of haemagglutinated chick cells could be observed within 30-45 min of agglutination.

In this laboratory, the chick cells have been used in the IHA with equal sensitivity and specificity for demonstration of specific serum antibodies in the diagnosis of amoebiasis, echinococcosis and filariosis (Parija, 1985; Parija, 1986; Parija *et al.*, 1989). Therefore, the present study was carried out to evaluate the use of chick cells in the IHA for the diagnosis of neurocysticercosis.

Materials and Methods

Patients: Patients attending epilepsy clinic, JIPMER Hospital, Pondicherry were included in the present study. The following groups of the patients and controls were included:

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Group I: It included 15 cases of neurocysticercosis with single cystic lesion in the brain on CT scan; Group II: it included 15 cases of neurocysticercosis with multiple small cystic lesions in brain on CT scan; Group III: included 15 cases of neurological disorders (meningitis, etc.) other than cysticercosis; and Group IV: included 30 normal healthy people as controls.

Blood samples were collected from these cases and controls, sera were separated and stored at -20°C till use.

Cysticercus antigen: Cysticercus cellulosae cysts, from infected pig muscles were collected for antigen. Preparation of complete homogenate. Cysticercus cellulosae antigen was prepared as per the procedure given by Sreenivasamurthy *et al.* (1999). Briefly, cysts were homogenized in a glass tissue homogenizer with phosphate buffered saline (PBS, pH 7.2) containing phenyl-methyl sulphonyl fluoride (PMSF) (0.1 mM), the suspension was then sonicated 8 times (each time for 1 min duration) at 12 kH with 30 sec cooling interval; then centrifuged at 4°C for 30 min at 14,000 rpm. The supernatant was collected as the complete homogenate antigen and stored at -20°C.

Red blood cells (RBCs):

Chick RBCs: Blood was collected from chicks into Alsever's solution. The cells were washed thrice in PBS, pH 7.2, (0.15 M) and treated with the stabilizing agent within 48 h.

Human RBCs: Blood was collected from human group 'O' into Alsever's solution. The cells were washed thrice in PBS, pH 7.2, (0.15 M) and treated with the stabilizing agent within 48 h.

Stabilization of chick RBCs: Stabilization of the chick RBCs with different aldehydes was carried out as per the procedures described below:

Stabilization of chick RBCs with formaldehyde: Chick RBCs were stabilized by the method of Fulthrope *et al.* (1961) as cited by Herbert (1978). To a suspension of chick RBCs (10 ml of packed cells in 500 ml of PBS, pH 7.2). 50 ml of formalin (aqueous formaldehyde, 40% w/v) was added slowly from a burette, over a period of 30 min. The mixture was left overnight at 4°C and further 50 ml of formalin was then added in one lot. Twenty-four hours later the preserved cells were washed thrice in PBS, pH 7.2 and stored at 4°C as a 50% v/v suspension in a 1 in 200 dilution of formalin in PBS, pH 7.2.

Stabilization with glutaraldehyde: The RBCs were stabilized with glutaraldehyde as per the method described by Avermeas *et al.* (1969) as cited by Parija *et al.* (1986). Briefly, 10 ml of 5% v/v aqueous glutaraldehyde was added to 1 ml of 10% suspension of chick RBCs in PBS, and the mixture was stirred by a magnetic stirrer for 4 h as a 30% v/v suspension in PBS.

Stabilization with pyruvic aldehyde: The RBCs were stabilized by pyruvic aldehyde as per the method described by Parija *et al.* (1986). A reaction mixture consisting of the following substances: 1.7% NaCl, 12 ml; 40% v/v aqueous pyruvic aldehyde, 4 ml; 1% Na_2Co_3 35 ml; Soresen's phosphate buffer (pH 7.2) 7 ml added in the order given. The reaction mixture thus prepared was kept in the cold and to this 10 ml of a 50% suspension of chick RBCs was added. The mixture was stirred for 30 min and stored for 24 h at 4°C, the cells were washed thrice in PBS and stored in this as a 2.5% v/v suspension at 4°C.

Double aldehyde stabilization (DAS) with pyruvic aldehyde and glutaraldehyde: Double aldehyde stabilization (DAS) of chick RBCs was carried out, as per the method described by Parija and Ananthakrishnan (1985). The cells were first stabilized with pyruvic aldehyde (as above). To a 2.5% suspension of these an equal volume of cold tannic acid (1 in 25,000 in PBS, pH 7.2) was added and the mixture kept at 4°C for 30 min. The cells were then washed thrice in PBS and made upto a 4% suspension in the same diluent. To this was added an equal volume of 2% v/v aqueous glutaraldehyde in PBS, pH 7.2 over a magnetic stirrer which was run for 2 h. The cells were then washed thrice with PBS, pH 7.2 and stored as a 50% v/v suspension in this diluent.

Sensitization of double aldehyde stabilized chick cells with parasite antigen: The DAS chick cells were sensitized with the cysticercus antigen, as described by Farshy and Kagan (1972). Briefly, one volume of packed DAS cells was suspended in 10 volumes of an optimum sensitizing dose (OSD) of the antigen prepared in PBS, pH 6.4. The OSD determined for the particular batch of antigen was a dilution 1:80 in PBS, pH 6.4. The cell plus antigen mixture was incubated at 50°C in water batch of antigen for 5 min. The sensitized cells were washed thrice with PBS, pH 7.2 and stored in small volumes as a 10% suspension in PBS, pH 7.2 at 4°C until used.

Sensitization of single aldehyde stabilized chick cells with parasite antigen: Single aldehyde stabilized chick cells were first tanned, as described above (in case of DAS) just before to sensitization. Then rest of the procedure for sensitization was same as per the DAS cells.

Stabilization and sensitization of human 'O' RBCs: The human 'O' RBCs were stabilized by double aldehyde stabilization methods in the same way as described for chick

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RBCs. The stabilized human 'O' RBCs were sensitized with the OSD of cysticercus antigen in the same ways as described for the stabilized chick RBCs. The sensitized cells were stored in small volumes as a 10% suspension in PBS, pH 7.2 at 4°C until used.

Indirect haemagglutination test: Stored sensitized chick RBCs were used to prepare a 1% suspension in PBS, pH 7.2 bovine serum albumin (BSA) (0.1%). This suspension was used in the IHA test on the same day and after storage at 4°C for 7, 14, 31 and 45 days. The test was performed as described by Parija and Ananthakrishnan (1985). The results were read by observing the settling patterns of the cells in the plate after incubation for 15, 30, 45, 60 and 120 min at room temperature and after holding the plates overnight at 4°C.

The IHA employing the stored sensitized DAS human RBCs were carried out on the same batch of sera for demonstration of serum antibodies.

Results and Discussion

Agglutinated chick RBCs settled after 30 to 45 min of incubation at room temperature and demonstrated a definite pattern of haemagglutination with the cysticercus positive sera. A titer of >64 was considered to be diagnostic titer of cysticercosis.

Table 1 shows the IHA times obtained with the sera of cases and controls by employing four types of stabilized chick RBCs. In case of most of the sera from patients with cysticercosis, the IHA using DAS chick cells gave 204 times higher titer than the single aldehyde stabilized chick cells. Table 2 shows the comparative evaluation of the IHA using chick DAS cells and human 'O' RBCs on the sera from the cases and controls. No statistical difference was noted in the titers between the IHA using DAS chick cells and the IHA using human 'O' DAS cells.

Neurocysticercosis, a parasitic disease of central nervous system, is an important cause of epilepsy and morbidity in tropical countries. The infection is acquired by ingestion of vegetables and water contaminated with human excreta containing eggs from carriers of adult *Taenia solium*. Occasionally, infection also occurs by regurgitation of eggs of *T. solium* or auto-infection by the faecal-oral route in patients harbouring the adult parasite. Epilepsy is the commonest feature of cysticercosis in brain; other less common feature includes raised intracranial pressure meningoencephalitis and spinal or ocular cysticercosis. To know the prevalence of the disease on epidemiological surveys, there is need for an inexpensive test like IHA.

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Non-nucleated RBCs from sheep (Parija and Ananthakrishnan, 1985) and human of blood group 'O' (Herbert, 1978) and nucleated RBCs from turkey (Singh *et al.*, 1980) and goose (Mannen *et al.*, 1984) have been used successfully in IHA tests in various parasitic infections. Turkey and goose erythrocytes are usually recommended because they are nucleated, heavier, and thus settle quickly. The results can be read rapidly within 30-45 min using IHA test. Because goose and turkey RBCs are scarce in this part of India, the present study was performed to evaluate chick RBCs as alternative nucleated RBCs for the IHA test for cysticercosis. The chick cells as carrier of antigen have been used in IHA in hydatid disease (Rigano *et al.*, 2002; Abdel Aal *et al.*, 1996; Parija *et al.*, 1987), lymphatic filariosis (Sengupta *et al.*, 1986; Singh *et al.*, 1980) and amoebiasis (Ahsan *et al.*, 2002; Wiwanitkit *et al.*, 2002; Parija *et al.*, 1989), and in viral infections (Skaug *et al.*, 1975), however, the use of such cells in the IHA tests for cysticercosis is lacking. Hence, the present study was performed to evaluate the chick RBCs in the IHA for diagnosis of cysticercosis.

In the present study, haemagglutinated chick RBCs showed complete haemagglutination after 30 to 45 min of incubation with test sera at room temperature. This is a major advantage of chick RBCs over that of human RBCs, which take more than 6 h to settle and agglutinate. Moreover, heterophilic antigens were not a problem with chick cells and no prior absorption of the test sera was required, as is needed with non-nucleated sheep cells.

Since the cut-off titer for a diagnostic test including the IHA vary from place to place, the same for a particular geographical area is determined by screening a large number of apparently normal population sera for parasite specific antibodies and taking a mean average titer of sera ± 2SD. Before the present study was initiated, sera from more than 1000 apparently normal sera were screened for cysticercus antibodies and after analysis, >1:64 was considered as a cut-off titer for anti-cysticercus antibodies for population residing in and around Pondicherry. At this titer and above, a total of 24 sera out of 30 sera from the cases of neurocysticercosis (Groups I & II) were positive for cysticercus antibodies by the IHA employing the chick DAS as well as human DAS cells sensitized with cysticercus antigen.

Results of the present study show that the use of chick DAS cells in comparison to single aldehyde stabilized chick cells greatly increased the sensitivity of the IHA tests (Table 1). The IHA employing DAS chick cells was found to be equally sensitive with that of the IHA using DAS

Table 1. Titers of IHA in patients' sera using chick RBCs stabilized with various aldehydes

No.	FA	GA	PA	PA+GA	No.	FA	GA	PA	PA+GA
Group I					**Group III**				
01	1:2	1:2	1:2	1:2	31	1:32	1:64	1:64	1:128
02	1:16	1:4	1:32	1:256	32	1:32	1:32	1:8	1:32
03	1:64	1:32	1:32	1:64	33	1:8	1:8	1:8	1:8
04	1:2	!:8	!:64	1:64	34	1:16	1:32	1:32	1:32
05	1:2	1:32	1:16	1:128	35	1:32	1:64	1:32	1:64
06	1:32	1:64	1:64	1:128	36	1:2	1:8	1:4	1:8
07	1:4	1:8	1:16	1:64	37	1:4	1:8	1:4	1:16
08	1:8	1:8	1:32	1:64	38	1:8	1:8	1:8	1:16
09	1:16	1:64	1:16	1:256	39	1:8	1:8	1:4	1:8
10	1:16	1:16	1:32	1:32	40	1:16	1:8	1:32	1:32
11	1:32	1:64	1:64	1:128	41	1:8	1:8	1:4	1:8
12	1:2	1:4	1:2	1:4	42	1:16	1:16	1:8	1:8
13	1:32	1:32	1:64	1:128	43	1:2	1:4	1:2	1:4
14	1:2	1:8	1:4	1:8	44	1:4	1:8	1:4	1:32
15	1:4	1:8	1:8	1:8	45	1:16	1:16	1:8	1:32
Group II					**Group IV**				
16	1:8	1:8	1:8	1:64	46	1:4	1:4	1:16	1:16
17	1:32	1:16	1:16	1:128	47	1:4	1:4	1:16	1:16
18	1:16	1:64	1:16	1:64	48	<1:2	1:2	1:8	1:16
19	1:16	1:64	1:32	1:128	49	1:4	1:4	1:8	1:16
20	1:8	1:16	1:8	1:64	50	1:2	1:4	1:8	1:8
21	1:16	1:64	1:32	1:256	51	1:4	1:4	1:4	1:16
22	1:16	1:16	1:8	1:64	52	1:2	1:8	1:4	1:8
23	1:4	1:8	1:8	1:128	53	1:2	1:16	1:16	1:32
24	1:16	1:8	1:32	1:128	54	1:2	1:4	1:2	1:8
25	1:32	1:64	1:32	1:128	55	1:2	1:4	1:8	1:16
26	1:16	1:64	1:16	1:64	56	1:2	1:2	<1:2	1:16
27	1:32	1:16	1:8	1:64	57	<1:2	<1:2	1:8	1:16
28	1:32	1:32	1:32	1:128	58	<1:2	<1:2	1:4	1:16
29	1:2	1:8	1:4	1:8	59	1:2	1:2	1:2	1:2
30	1:32	1:16	1:64	1:64	60	<1:2	<1:2	1:8	1:4

FA=Formaldehyde; GA=Glutaraldehyde; PA=Pyruvic aldehyde

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human RBCs (Table 2). The IHA antibody titer of the sera from the known cases of single cystic lesions of NCC (Group I) by using DAS cells were more or less similar to that using single aldehyde stabilized cells (Tables 1 and 2). This may be due to the actual low titer of the cysticercus antibodies in sera which can not be detected even by using the DAS cells. It is known that in case of neurocysticercosis with single cystic lesion in brain, it may evoke poor antibody response unlike in case of multiple cysts. This is further supported by the observation in the present study that there was a noted difference in the antibody titers detected by the IHA using DAS cells in comparison to that using single aldehyde stabilized cells in the sera collected from the known cases of NCC with multiple cystic lesions (Group II) (Tables 1 and 2).

In the present study, the IHA using DAS cells showed a false positivity of 13.3% with sera collected from the cases of neurological disorders other than cysticercosis (Group III). Two sera from this group showed a diagnostic IHA titre (1:64 and 1:128). This false positivity may be due to non-specific reaction or other unknown factors. It is well recognized that sera from the cases of tubercular meningitis may show cross reaction with cysticercus antigens (Katti, 2002). But in the present study, the CSF from these

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Table 2. Comparative titres of IHA on sera from suspected cases of cysticercosis using DAS human and DAS chick RBCs

Serum No.	Human 'O' RBCs	Chick RBCs	Serum No.	Human 'O' RBCs	Chick RBCs
Group-I			**Group-III**		
01	1:4	1:2	31	1:64	1:128
02	1:256	1:256	32	1:32	1:32
03	1:64	1:64	33	1:4	1:8
04	1:64	1:64	34	1:32	1:32
05	1:128	1:128	35	1:32	1:64
06	1:64	1:128	36	1:8	1:8
07	1:128	1:64	37	1:32	1:16
08	1:64	1:64	38	1:16	1:16
09	1:128	1:256	39	1:8	1:8
10	1:32	1:32	40	1:8	1:32
11	1:128	1:128	41	1:4	1:8
12	1:4	1:4	42	1:8	1:8
13	1:128	1:128	43	1:4	1:4
14	1:8	1:8	44	1:8	1:32
15	1:8	1:8	45	1:16	1:32
Group-II			**Group-IV**		
16	1:64	1:64	46	1:16	1:16
17	1:128	1:128	47	1:32	1:16
18	1:128	1:64	48	1:8	1:16
19	1:128	1:128	49	1:16	1:16
20	1:64	1:64	50	1:8	1:8
21	1:128	1:256	51	1:16	1:16
22	1:64	1:64	52	1:16	1:8
23	1:128	1:128	53	1:16	1:32
24	1:128	1:64	54	1:8	1:8
25	1:128	1:128	55	1:8	1:16
26	1:64	1:64	56	1:8	1:16
27	1:64	1:64	57	1:8	1:16
28	1:64	1:128	58	1:4	1:16
29	1:16	1:8	59	1:2	1:2
30	1:64	1:64	60	1:4	1:4

two cases were also negative for acid-fast bacilli (AFB) by smear and culture.

The results of the present study show that the IHA using DAS chick RBCs instead of human 'O' RBCs will be a useful procedure with equal sensitivity for the diagnosis of neurocysticercosis. The IHA test employing chick red blood cells shows great promise in the diagnosis of porcine cysticercosis.

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