Detection of antibodies to *Blastocystis hominis* in immunised mice using counterimmunoelectrophoresis (CIE) and enzyme-linked immunosorbent assay (ELISA)

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Abstract

Blastocystis hominis can cause diarrhoea in both immunocompetent and immunocompromised patients. Laboratory confirmation of the infection is usually based on detection of the parasite directly in stools or after *in vitro* culture. As these may not detect all cases, various immunological and molecular tests are being developed to increase the sensitivity and specificity of laboratory detection of infection. The indirect fluorescence antibody assay (IFA), counterimmunoelectrophoresis (CIE) and enzyme-linked immunosorbent assay (ELISA) are being evaluated for the detection of parasite antigens or specific *B. hominis* antibodies. The present study seeks to determine if homologous and heterologous antigens can be used in the CIE and ELISA to detect antibodies in mice immunised differently with an axenic *B. hominis* isolate (isolate C), xenic isolates (isolate H7 and RN), and an *Escherichia coli* isolate (Bac2). The granular form of the parasite isolates was used for antigen preparation. In the CIE, sera of mice immunised with isolate C reacted only with antigen C but not with H7, RN or Bac2 antigens. However, antigen C reacted with sera of all mice immunised with isolate RN, 37.5% of those immunised with H7, but not Bac2. Sera of mice immunised with RN and H7 all reacted with RN, H7 and Bac2 antigens. It is believed that the cross-reactions of sera from mice immunised with the xenic *B. hominis* isolates are due to antibodies against *E. coli*. Mean antibody levels detected in the ELISA with homologous and heterologous antigens followed closely the CIE results. Immune sera against isolate C had significantly higher OD levels in the ELISA with antigen C at week 2 to 6 post-immunisation than sera of mice immunised against the xenic isolates (RN and H7) and Bac2 ($P < 0.005$). ELISA OD levels in sera of mice immunised with xenic isolates were higher with homologous antigens. OD values were higher against other xenic isolate antigens and Bac2 antigen than with isolate C. All antigens induced significantly higher antibody levels than pre-immunisation sera in the sera of immunised mice at week 2 to 6 post-immunisation ($P < 0.005$). It is concluded that both the CIE and ELISA can be used to detect *B. hominis* antibodies. It is recommended that the antigen for these assays be from axenic isolates of *B. hominis* to avoid cross-reactions with antibodies directed against *E. coli*. The CIE is technically simpler and quicker to carry out than the ELISA but uses more antigens.

Key words: *Blastocystis hominis*; antibodies; ELISA; CIE

Introduction

Blastocystis hominis is a very common parasite of humans and animals and recent identification of the cyst stage suggests an oral-faecal route of transmission (Borebam & Stenzel, 1993a & b). The various morphological forms of *B. hominis* are vacuolar, granular, amoeboid, cyst, avacuolar and multivacuolar (Stenzel & Boreham, 1996).

Infections have been reported worldwide affecting both immunocompetent and immunodeficient individuals (Boreham & Stenzel, 1996). It occurs most commonly among immunocompromised patients (Cegielski *et al.*, 1993; Garavelli *et al.*, 1988; Garcia *et al.*, 1984; Rolston *et al.*, 1989).

Although its pathogenic role in humans has not been established, the parasite can cause intestinal pathology

in experimentally infected Balb/c mice (Moe *et al.*, 1997). Furthermore, anti-*Blastocystis* antibodies were found to be associated with irritable bowel syndrome (Hussain *et al.*, 1997). Studies using endoscopy and biopsy have indicated that *B. hominis* does not invade the colonic mucosa in human patients (Dawes *et al.*, 1990; Dellers *et al.*, 1992; Diaczok & Rival, 1987; Doyle *et al.*, 1990; Garavelli *et al.*, 1992a; Kain *et al.*, 1987; Tsang *et al.*, 1989; Zuckerman *et al.*, 1990). Oedema and inflammation of intestinal mucosa may be present (Garavelli *et al.*, 1991; Garavelli *et al.*, 1992a; Kain *et al.*, 1987; Russo *et al.*, 1988; Zuckerman *et al.*, 1994), with colonic ulceration and *B. hominis* organisms in the superficial lamina propia and gland spaces (Al-Tawil *et al.*, 1994).

It has also been suggested that *B. hominis* can cause toxic-allergic reactions, leading to non-specific inflammation of colonic mucosa (Garavelli *et al.*, 1991; Garavelli *et al.*, 1992a). Zierdt (1991) based on studies involving isolated segments of the rabbit ileum, suggested the presence of a toxin fraction in the *B. hominis* culture medium. Zuckerman *et al.* (1994) using an excreted radioactive marker, reported impaired intestinal permeability in patients with *B. hominis* infection.

Zierdt *et al.* (1995) reported that ELISA could potentially be used to detect antibodies in sera of *Blastocystis* infected patients. Garavelli *et al.* (1992b) successfully used the immunofluorescence assay and enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to *B. hominis* in four patients. An ELISA method using *B. hominis* antigens (strain 91 isolated from an acute symptomatic human infection) was able to detect IgG antibodies in sera of 28 out of 30 symptomatic patients with no enteric protozoan pathogens other than *B. hominis* (Zierdt *et al.*, 1995). Lim *et al.* (1997) reported that antibodies produced in mice against *B. hominis* reacted only with the homologous antigen. No cross-reactivity was observed with *Giardia lamblia* and *Entamoeba histolytica*.

The counterimmunoelectrophoresis (CIE) has also been widely used for the detection of antigen in serum, or other body fluids for the diagnosis of various infections (Greenwood *et al.*, 1971: Parija *et al.*, 1997). It has been used previously for the detection of anti-*Entamoeba* antibodies and circulating amoebic antigen in serum for the diagnosis of amoebic liver abscess. Rabbit antisera to unheated whole-cell *B. hominis* antigen reacted with vacuolar, granular and amoeboid forms of the parasite (Zierdt, 1991).

The objectives of the present study were to determine whether the CIE could be used to detect and characterise specific antibodies to *B. hominis* antigens in Balb/c mice experimentally immunised with soluble antigens of pathogenic and non-pathogenic isolates of the parasites. We also compared the use of soluble antigens from different parasite isolates in the CIE and ELISA.

Materials and Methods

Xenic and axenic cultures of B. hominis

B. hominis parasites were isolated from stools of patients with diarrhoea. The parasites (isolate RN and H7) were cultured in Jones medium with 10% horse serum. Subcultures were carried out daily.

Axenic *B. hominis* (isolate C) was cultured in complete IMDM medium with 10% horse serum at 37°C in an anaerobic jar.

Harvesting and preparation of B. hominis antigen

Each xenic (RN and H7) and axenic (C) parasite culture was centrifuged at 12,000 rpm at 4°C for 5 minutes. The supernatant was discarded and the pellets were transferred to a new sterile test tube followed by addition of 10 ml of sterile 0.85% NaCl normal saline and then centrifuged. The washing was repeated five times. The washing could be repeated further to discard as much bacteria as possible in the culture. The pellet, which contained a large number of *B. hominis* and very small amount of bacteria (isolates RN and H7) was collected and kept at 4(C until use.

The pellets were sonicated to obtain soluble antigens. The sonication was repeated ten times, 1 min. each time and at 1 min. intervals. After incubation at 4°C overnight, the preparation was centrifuged at 12,000 rpm at 4°C for 20 min. The pellet was discarded and the supernatant filtered through a 0.2 µm sterile filter, aliquoted, and kept at -20°C until use.

Culture and preparation of Bac2

Escherichia coli isolated from the culture of *B. hominis* isolate RN was used in this study. The culture of Bac2 and preparation of its soluble antigen are as described above for xenic culture of *B. hominis*.

Immunisation of the Balb/c mice

Five groups of 10, four to six weeks old inbred Balb/c mice were used. Four groups were immunised using isolates RN, H7, C and *E. coli* (Bac-2) antigen. The fifth group served as control.

The pre-immunisation sera were obtained from blood through tail bleed. Each mouse was immunised with three doses of antigen subcutaneously, each dose

given at two weekly intervals. Each dose consisted of 0.5 ml antigen in 0.5 ml normal saline (the final protein concentration being 100-200 µg / ml) emulsified in an equal volume (1 ml) of Freund's complete Adjuvant (FCA).

Each mouse was given 1 ml emulsified antigen at two different sites on the dorsal aspect. Sera were collected at 2 weeks post-immunisation through tail bleed after the 1st and 2nd immunisation and by cardiac puncture of the mice at autopsy. Antibody levels were determined by using CIE and ELISA.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA method used was that of Voller *et al.* (1976), which is a microplate modification of the method of Engvall & Perlmann (1972). The method used in this study was the indirect assay of antibodies using the horseradish peroxidase enzyme system. A checkerboard titration for antigens was performed with positive and negative control sera.

One hundred µl of soluble antigens diluted 1:100 in coating buffer were added to each well of microtitre ELISA plate (Immulon II, Dynatech Laboratories, USA), and left overnight at 4°C for coating to occur.

The plate was washed three times with PBS with 0.05% Tween 20 (PBS-Tween) solution, pH 7.4. After drying, the test serum diluted 1:50 with 5% skimmed milk in PBS-Tween was added to duplicate wells. The plate was covered and incubated for 2 hours at room temperature.

After washing as above, 100 µl of peroxidase conjugated polyvalent goat anti-mouse immunoglobulins (GIBCO) diluted 1:1000 in 5% skimmed milk in PBS-Tween (predetermined optimal dilution determined by checkerboard titration) were added to each well. The plate was left in a clean container for 3 hours at room temperature followed by washing as before.

One hundred µl of the enzyme substrate (orthophenylenediamine) was then added to each well and the plate was incubated in the dark for 20 minutes at room temperature.

The enzyme reaction was stopped by adding 50 µl of 2.5M Sulphuric acid to each well. The samples were read using an ELISA reader (Dynatech Laboratories, USA) at 492 nm and the optical density (OD) values at E492 nm recorded. The mean OD readings in duplicate wells were taken. Known positive and negative control sera were included in each plate.

Counter-immunoelectrophoresis (CIE)

The gels were prepared on 80 x 100 mm glass slides with 15% Agarose gel (0.26 g in 20 ml of immunoelectrophoresis (IEP) buffer, pH 8.6). The agar was evenly distributed on the slide by carefully spreading it over the glass surface with a pipette tip.

Forty pairs of wells, 3 mm in diameter and 3 mm apart were punched out on the gel with a template. Each well was filled with 10 µl of the appropriate reactant. The well containing antigens were placed on the cathodic side of electrophoresis chamber and the serum (antibodies) on anodic side. The electrophoresis chamber was filled with IEP buffer (2.5M, pH 8.6) and a current of 40V for 90 minutes was used.

Immediately after completion of electrophoresis the slides were read unstained with oblique lighting against a black background. The slides were then stained with amido black for 30 minutes.

Excess stain was removed by soaking in tap water. The precipitin bands appeared dark blue in colour.

Statistical analysis

ELISA OD readings were analysed using SPSS® Statistical Software Package, Version 7.5. Mean and SD of OD values were calculated and differences in means between and among groups of mice immunised with the various antigens were analysed using ANOVA. Statistical significance was set at $P < 0.05$.

Results

Three different isolates of *B. hominis* (isolate C, RN and H7) and *E. coli* (Bac2) grown either in the Jones medium or IMDM medium were used in this study. They were cultured in axenic or xenic conditions. The axenic culture was carried out in an anaerobic chamber at 37°C and the xenic cultures containing bacteria were cultured at 37°C. The granular form of *B. hominis* predominated and was easier to grow in culture. This parasite form was used as the source of antigen in the CIE as well as ELISA for detection of specific antibodies.

In the ELISA, *B. hominis* isolate C antigen reacted well with immune sera produced against isolate C antigen in mice. There was cross reactivity against RN (xenic) antigens, the OD values attained being higher than that with the homologous antigen. Very low cross-reactivity was observed with isolate H7 and Bac2 antigens. Antibody levels were detected in mice at week 2 after the first immunisation, rose rapidly, and by 2 weeks after the third immunisation dose (i.e. at week 6 of observation), the mean ± SD was 0.62 ± 0.10 (Table 1).

Table 1. Mean ± SD optical density (OD at 492 nm) readings in mice immunised with *Blastocystis hominis* isolate C in the enzyme-linked immunosorbent assay with various antigens

Antigen	Mean ± SD OD readings at immunisation week*			
	0	2	4	6
B. hominis (C)	0.06 ± 0.01	0.53 ± 0.07	0.57 ± 0.07	0.62 ± 0.10
B. hominis (H7)	0.01 ± 0.00	0.01 ± 0.00	0.04 ± 0.02	0.14 ± 0.06
B. hominis (RN)	0.14 ± 0.01	0.56 ± 0.13	0.71 ± 0.20	0.90 ± 0.29
Escherichia coli (Bac2)	0.01 ± 0.00	0.13 ± 0.07	0.19 ± 0.07	0.28 ± 0.06

*Week 0, 2, 4, & 6 = pre-immunisation, and 2 weeks after the 1st, 2nd and 3rd immunisation doses given at 2 weekly intervals respectively.

Table 2. Mean ± SD optical density (OD at 492 nm) readings in mice immunised with *Blastocystis hominis* isolate H7 in the enzyme-linked immunosorbent assay with various antigens

Antigen	Mean ± SD OD readings at immunisation week*			
	0	2	4	6
B. hominis (C)	0.06 ± 0.00	0.13 ± 0.07	0.21 ± 0.06	0.26 ± 0.07
B. hominis (H7)	0.01 ± 0.00	0.05 ± 0.02	0.36 ± 0.12	0.49 ± 0.07
B. hominis (RN)	0.14 ± 0.01	0.20 ± 0.07	0.86 ± 0.19	1.17 ± 0.12
Escherichia coli (Bac2)	0.01 ± 0.00	0.25 ± 0.09	0.60 ± 0.12	0.80 ± 0.15

*Week 0, 2, 4, & 6 = pre-immunisation, and 2 weeks after the 1st, 2nd and 3rd immunisation doses given at 2 weekly intervals respectively.

Sera of mice immunised with H7 antigens reacted with antigens of both xenic isolates (H7 and RN) as well as Bac2 (Table 2). There was little cross-reactivity with antigens of the axenic isolate C. Maximum OD readings were attained 2 weeks after the third immunisation dose, this being 0.49 ± 0.07 and 1.17 ± 0.12 with homologous (H7) and RN antigens respectively.

Sera of mice immunised against RN antigen reacted strongly against the homologous antigen, the highest OD reading (1.91 ± 0.16) being 2 weeks after the third immunising dose (Table 3). There was cross reaction with Bac2 antigen, the OD values at 2 weeks after the third immunisation dose being 0.72 ± 0.14. There was slight cross reactivity with antigens of isolate C and H7.

Mice immune sera against Bac2 antigens reacted strongly against RN and Bac2 antigens, the OD readings being highest at 2 weeks after the third immunisation dose (Table 4). These OD readings were higher with RN (1.09 ± 0.48) than the homologous antigen Bac2 (0.79 ± 0.16). There was little cross reactivity with antigens of isolate C and H7.

Table 3. Mean ± SD optical density (OD at 492 nm) readings in mice immunised with *Blastocystis hominis* isolate RN in the enzyme-linked immunosorbent assay with various antigens

Antigen	Mean ± SD OD readings at immunisation week*			
	0	2	4	6
B. hominis (C)	0.06 ± 0.00	0.09 ± 0.02	0.14 ± 0.00	0.19 ± 0.05
B. hominis (H7)	0.01 ± 0.00	0.04 ± 0.02	0.18 ± 0.03	0.27 ± 0.03
B. hominis (RN)	0.14 ± 0.01	0.77 ± 0.22	1.59 ± 0.19	1.91 ± 0.16
Escherichia coli (Bac2)	0.01 ± 0.00	0.20 ± 0.13	0.48 ± 0.14	0.72 ± 0.14

*Week 0, 2, 4, & 6 = pre-immunisation, and 2 weeks after the 1st, 2nd and 3rd immunisation doses given at 2 weekly intervals respectively.

Table 4. Mean ± SD optical density (OD at 492 nm) readings in mice immunised with *Escherichia coli* isolate Bac2 in the enzyme-linked immunosorbent assay with various antigens

Antigen	Mean ± SD OD readings at immunisation week*			
	0	2	4	6
B. hominis (C)	0.06 ± 0.00	0.06 ± 0.00	0.08 ± 0.00	0.11 ± 0.00
B. hominis (H7)	0.01 ± 0.00	0.04 ± 0.01	0.13 ± 0.04	0.20 ± 0.00
B. hominis (RN)	0.15 ± 0.02	0.28 ± 0.12	0.71 ± 0.43	1.09 ± 0.48
Escherichia coli (Bac2)	0.02 ± 0.01	0.28 ± 0.16	0.56 ± 0.15	0.79 ± 0.16

*Week 0, 2, 4, & 6 = pre-immunisation, and 2 weeks after the 1st, 2nd and 3rd immunisation doses given at 2 weekly intervals respectively.

The CIE test using homologous and heterologous antigens was used to detect antibodies in immune sera of mice immunised with the various *B. hominis* isolates (RN, C, and H7) and Bac2. The optimum running condition for the CIE was a current voltage of 40V for 90 min.

Immune sera produced against isolate C antigen only reacted with isolate C antigen (forming 1 precipitin band). Nine out of the ten (90%) immunised mice produced sufficient antibodies at week 6 post-immunisation to give a positive result in the CIE. There was no cross-reaction with heterologous antigens (no precipitin band with isolates H7, RN, and Bac2 antigens).

Immune sera produced against isolate H7 antigen reacted with the homologous antigen (isolate H7 antigen) and also cross-reacted with isolates RN and Bac2 antigens. Of the sera from the 8 H7 immune mice remaining at week 6 post-immunisation, 3 (37.5%) reacted with antigen C, but all 8 (100%) reacted with antigens H7, RN and Bac2. However there was weak cross-reaction with isolate C antigen, and only 3 out of 8 mice sera showed precipitin bands with it.

Immune sera produced against isolate RN antigen reacted with the homologous antigen and also cross-reacted with antigens of isolates H7, C and Bac2. All the sera from the 10 mice at week 6 post-immunisation showed a precipitin band in the CIE with all the antigens tested.

Immune sera produced against isolate Bac2 antigen reacted with the homologous antigen (isolate Bac2 antigen). Only 4 out of 10 sera showed precipitin bands. There was also cross-reaction with isolate H7 and RN antigens, 20% and 10% showing precipitin bands respectively. None reacted with antigen of the *B. hominis* axenic isolate C. Sera of control mice did not react with any of the *B. hominis* or Bac2 antigens.

Control mice sera did not form any precipitin band (no reaction) with the all isolate antigens tested.

Discussion

The present study was carried out to assess the use of the CIE and ELISA in detecting *B. hominis* antibodies in Balb/c mice experimentally immunised with an axenic isolate (isolate C), two xenic isolates (isolates RN and H7) of *B. hominis*, and *Escherichia coli* (isolate Bac2). The latter is a common contaminant in xenic cultures.

The ELISA could detect antibodies in immunised mice as early as week 2 after the first immunisation dose. There was in general, a progressive increase in the antibody levels as detected by the ELISA in all immunised mice in the subsequent weeks until autopsy at week 6 or two weeks after the third unmunising dose. All antigens induced significantly higher antibody levels than pre-immunisation sera in the sera of immunised mice at week 2 to 6 of immunisation ($P < 0.005$).

In general, the ELISA using *B. hominis* antigens detected higher antibody levels in immune sera when the antigen used was homologous with the immunising antigen (Tables 1-4). Immune sera against isolate C had significantly higher OD levels in the ELISA with antigen C at week 2 to 6 of immunisation than sera of mice immunised against RN, H7 and Bac2 antigens ($P < 0.005$).

Sera of mice immunised with *B. hominis* isolates H7 and RN cross-reacted more intensely with antigens prepared from isolates cultured in the presence of *E. coli* (i.e. xenic isolates H7 and RN). It is postulated that this is due to cross-reactions with bacteria antigens as well as the presence of common antigenic epitopes in these xenic isolates.

Results obtained with the CIE showed some interesting results. Mice immunised with the axenic *B. hominis* isolate (isolate C) produced antibodies, which reacted

only with antigen C and not with the xenic isolates RN and H7 antigens. These sera also did not react with *E. coli* isolate Bac2 antigen. In contrast, sera from mice immunised with the xenic *B. hominis* isolates (isolates H7 and RN) reacted with antigens of the axenic and xenic isolates as well as Bac2. Sera of RN immune mice showed cross-reaction with all the antigens tested. Sera of mice immunised against Bac2 reacted with antigens of the *B. hominis* xenic isolates (H7 and RN) as well as with Bac2.

The differences in the CIE patterns obtained with immune sera against the various *B. hominis* and *E. coli* antigens necessitate some explanation. It is postulated that antibodies in mice immunised with isolate C are highly specific and only recognise antigen C. Antibodies in mice immunised with the xenic isolates H7 and RN cross-reacted with antigens of all 3 *B. hominis* isolates as well as Bac2. The cross-reactions are in part due to development of antibodies to *E. coli* present in xenic cultures. H7 and RN isolates probably share many common antigenic epitopes and also share some epitopes with isolate C. In view of these cross-reactions, it will be advisable to use antigen from an axenic isolate like C for the CIE.

In comparison to the ELISA, the CIE test was less sensitive. Lim *et al.* (1997) tested mice hyperimmune sera against *B. hominis*, *G. lamblia* and *E. histolytica*, with homologous and heterologous antigens in the ELISA. They found that antibodies produced against each parasite were specific and had very low ot no cross-reactivity with heterologous antigens. This indicates that the ELISA could be developed as a specific diagnostic assay for *Blastocystis* infections, even when the other two protozoan infections were present in the same individual.

Unlike the CIE, the ELISA results provide some quantification of antibodies in the immune sera. Higher levels of antibodies present are reflected in higher OD values. A positive result in the CIE is seen as the presence of precipitin bands and quantification based on the intensity of these bands is unreliable.

The CIE has some practical advantages over the ELISA. It is easy to read the results even with the naked eye whereas results of the ELISA are best read with an ELISA reader. The CIE is simple to set up and perform even when a small number of serum samples are to be tested. The ELISA is best carried out when there are many samples to be processed as in screening programmes. In the laboratory, which is capable of producing its own antigen, the cost of setting up the CIE test is much less than that of ELISA.

From the present study it is concluded that both the ELISA and the CIE can be used for the detection of antibodies to *B. hominis*. The CIE is only moderately sensitive compared to ELISA, which is sensitive and allows quantification of antibody levels. The CIE test is a rapid, simple test, which is inexpensive and can be set up in most diagnostic laboratories where a source of *B. hominis* is available. The ELISA is ideal for testing large numbers of samples as in a screening programme. It is recommended that the antigen for use in the ELISA should be from an axenic source of B. hominis to avoid cross-reactions with bacterial antigens.

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