Serologic Incidence of Cryptosporidial Infection in Broiler Flocks in Shiraz, Iran

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Summary

An antigen from the isolated propagated, and purified oocysts of *Cryptosporidium bailey* was prepared. To evaluate the serologic prevalence of *Cryptosporidium* in broiler flocks in Shiraz area a *Cryptosporidium*-specific indirect enzyme-linked immunosorbent assay (ELISA) was developed by using the antigen. Both the sensitivity and specifity of the ELISA was compared with an indirect immunofluorescent (IIF) test using the purified oocysts of the isolated *C.baileyi* as antigen. 125 out of 1522 (8.2%) serum samples from 26 broiler flocks were positive against *Crytosporidium*. Upon the result, previous reports on faecal shedding of *Cryptosporidium* in poultry farms was confirmed and *C.baileyi* can be added to the list of respiratory pathogens in Iran poultry industry.

***Key words*: *Cryptosporidium baileyi*, broiler, antigen, ELISA, IIF**

Introdution

Cryptosporidium spp. are small protozoan parasites that infect, grow and reproduce in epithelial cells which line portions of the gastrointestinal, respiratory and urinary tracts of vertebrates (Fayer 1997). In chickens two species of *Cryptosporidium* including *C.meleagridis* (Slavin 1955) and *C.baileyi* (Current *et al* 1986) are able to induce infection and disease (Current 1997,Fayer 1997,Goodwin 1989) .The first case of avian cryptosporidiosis in Iran reported without identification of the parasite species (Gharagozlu & khodashenas 1985). Some incidences of cryptosporidial infection in chicken faeces using modified Ziehl-Neelsen staining were reported (Badiyi 1990, Moradi 1998, Nikfarjam 1999, Noori *et al* 1994). Only in two reports

the parasite was identified as *C.meleagridis* (Noori *et al* 1994) and *C.baileyi* (Banani *et al* 1996). An indirect ELISA for detection of cyptosporidial antibodies in chicken, as a tool for serological survey of the infection, was developed in this study.

Materials and Methods

Sample. 1522 serum samples were collected randomly from 26 broiler flocks mainly at the slaughter time in Shiraz area. All chickens aged 20 to 60 days at the time of sampling, and 94% of them were more than 50-day-old. The sera stored at -20°C until use.

Parasite. Isolation and identification of *C.baileyi* from Shiraz broiler flocks has been described previously (Banani *et al* 1996). Antemortem and postmortem inspections of chicks in a 30-day -old broiler flock in Shiraz area revealed infectious bursal disease and cryptosporidial infection of cloaca and bursa of fabricius together .A modified Ziehl-Neelsen staining technique was used for initial diagnosis of *Cryptosporidium* were obtained by scraping bursa of fabricius from naturally infected birds. Oral inoculation of 2-day-old susceptible chicks with susceptible isolates confirmed the initial diagnosis. Light, fluorescent and electron microscopy also showed *Cryptosporidium* in the suspected tissue and samples. Based on the affinity of parasite to the respiratory tract, its high localization in the bursa of fabricius without any diarrhea in experimental infected birds, the size and shape of its oocysts, and also propagation of the parasite in embryonated eggs, it was concluded that the isolate was *C.baileyi*. This isolate was used to prepare antigen in immunoassay.

Prenaration of the ELISA antigen. Maintenance, propagation, and purification of oocysts of *C.baileyi* were performed as described by Lindsay *et al* (1986), Ungar *et al* (1986), Current (1990), and Hatkin *et al* (1993). Oocysts were maintained by passage through chicks. Faeces containing excreted oocysts were mixed with 2.5% potassium dichromate (w/v) and stored at 4°C. To purify oocysts, faeces were mixed with distilled water saturated with NaCl. This suspension was centrifuged at 1000 *g* and 4°C for 10 min, which resulted in a high concentration of oocysts in the supernatant. Supernatants were immediately diluted with water and again centrifuged at 1000 *g* at 4°C for 10 min, which resulted in a high concentration of oocysts in the pellet. Oocysts were separated and cleaned from remained faecal debris and bacteria by Curent's (1990) modification of Sheater's sugar flotation method and using 1%

sodium hypochlorite solution. Antigen was prepared from the purified oocysts by seven freeze-thaw cycles consisting of incubation in liquid nitrogen for 10 min, followed by incubation at 38°C until thawed (Current & Snyder 1988, Hathin *et al* 1993).

ELISA test. Fifty microlitres of *C.baileyi* antigen diluted in coating buffer (0.75g Na_2CO_3 and 1.46g $NaHCO_3$ in 500ml distilled water, pH9.6) were dispensed into each well of a 96-wellmicrotitre plate (Dynatech NR.M129B, UK) and allowed to adsorb to the plate overnight at 40°C. Fluid was aspirared from antigen coated target plates and a blocking solution (1% bovine serum albumin in wash buffer) was added and incubated for 15min a room temperature. Plates were then dumped, and all wells washed five times with washing buffer (0.05% Tween20 in PBS). Serum antibodies (IgG) directed against *C.baileyi* antigens were assayed by an ELISA procedure using peroxidase as enzyme and 0.04% orthophenylene diamine (OPD, Sigma) with 0.012% H_2O_2 in substrate buffer (0.2M Na_2HPO_4 and 0.1M citric acid) as substrate. The ELISA was adapted according to Dadrast (1989) and Ungar *et al* (1986). In this ELISA, specific goat anti-chicken IgG (FC) (Nordic immunologicals) as antiserum and rabbit anti-goat IgG (H+L) PO (Nordic immunologicals) as conjugate, diluted in dilution buffer (PBS 0.25% bovine serum albumin and 5% powdered skim milk) were used.

Optimum concentration dilution of antigen, serum, antiserum and conjugate were determined by checkerboard titration method. The incubation time and temperature for serum, antiserum and conjugate steps were 45min at 37°C and after incubation in each step by using the washing buffer, plates were washed and tapped dry five times. Substrate was added in the wells and incubated for 30min at 37°C.The substrate reaction was stopped using stop solution (2.5M H_2SO_4). The extent of hydrolysis of the substrate was measured by optical density (OD) at 490 nm with a microplate colorimeter (Mini reader II Dynatech laboratories Inc. Virginia, USA). SPF chicks (Lohmann, Germany) were infected with 10^6 *C.baileyi* oocysts isolated in Shiraz area, the infection was repeated to increase the immune response, and uninfected SPF or commercial broiler chicks were used as positive and negative controls, respectively. A specimen was considered positive if the OD from its well was greater than or equal to the mean + 2 SD of the OD of the negative controls.

Indirect immunofluoresecent (IIF) test. The IIF standardization and procedure were carried out by a modification of a previously described method (Current 1990, Current & Snyder 1988). 5×10^4 purified oocysts of isolated *C.baileyi* were coated and air dried onto each well of immunofluorescent slide as antigen. To fix the antigen we use mild heating. Test sera were added at a ten-fold dilutions beginning from 1:20 dilution and incubated in a moist chamber for 30min at room temperature. Then the slide was washed three times in PBS. In this IIF test goat anti-chicken IgG (Nordic immunologicals) diluted 1:80 in PBS and donkey anti-goat IgG (FITC) (Nordic immunologicals) of a 1:80 dilution were used and negative control sera were used in each test. Slides were mounted in 50% glycerine in distilled water and examined by fluorescence microscope (Leitz Wetzlar,Hund H500). In this test sera titer >1:40 were considered as positive and indicated of cryptosporidial infection.

Results

In this ELISA test optimum concentration of antigen was considered as fifty microlitres of *C.baileyi* antigen equivalent to 1.2×10^5 oocysts diluted in coating buffer for each well of the 96-well microtitre plate and the optimum dilution of test serum, antiserum and conjugate were determined 1:400, 1:1200 and 1:500 respectively .The OD of negative and positive control sera were 0.6-7.5 and 1.4-1.7 respectively, and the OD>0.86 was considered as positive .The sensivity, specifity and correlation rate of ELISA test in comparison with IIF test were 100%, 80.3% and 92.8% respectively. In this study 3 out of 26 (11.5%) broiler flocks and 125 of 1522 (8.2%) serum samples collected from those 26 flocks were positive against *Cryptosporidium*. The ages of all positive flocks were over 50 days posthatch. The contamination rates of three positive flocks were 27, 58 and 72%. These infected flocks were located in Ramjerd, Akbar-Abad and Kavar regions respectively.

Discussion

This is the first serological diagnosis of the cryptosporidial infection in Iran. This ELISA method can be used in human and other class of immunologbulin with some little modifications. For example coating obtained from *C.parvum* by purification methods of this study can be used for developing an ELISA for human, and by using goat anti-chicken IgA instead of goat anti-chicken IgG, local immunity in chicken
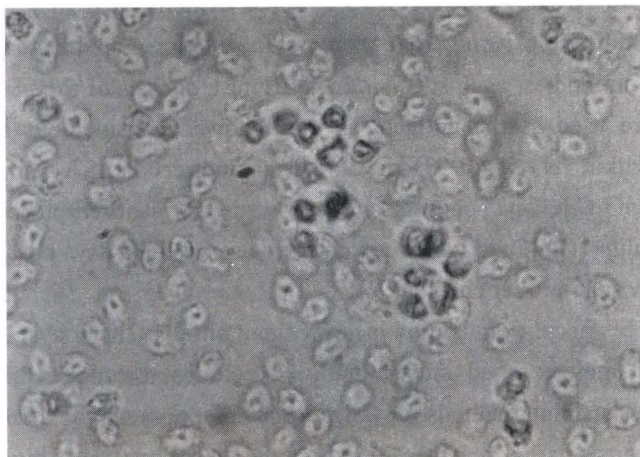
Figure 1. *Light microscope photomicrograph (x1100) of purified C.baileyi oocysts originally isolated from Shiraz broiler flocks in PBS, Banani et al (2000)*

against *Cryptosporidium* can be studied. This ELISA, because of cross-reaction between various species of *Cryptosporidium* even between *C.baileyi* and *C.parvum* (Current & Snyder 1988, Fayer 1997, Naciri *et al* 1994), can be detected antibody against chicken *Crytosporidium*.

Since there is no report about natural infection of birds with *Cryptosporidium* of other class of vertebrates like *C.parvum* in mammals, so positive rate in this study can be related to *C.baileyi* and, or *C.meleagridis* infection. But based on previous isolation of *C.baileyi* from Shiraz broiler flocks (Banani *et al* 1996), and other studies (Current 1997, Fayer 1997, Hatkin *et al* 1993, Naciri *et al* 1994, Snyder *et al* 1988) which indicate that *C.baileyi* is the most common species associated with infection and disease in broiler chickens, we can conclude that positive rate in this study primarily is due to *C.baileyi* infection.

Blagburn *et al* (1987) have shown a primary pathogenic role for *C.baileyi* by demonstrating in broiler chickens that intratracheal inoculation of oocysts resulted in severe respiratory infection, with some mortality. Several other reports of respiratory cryptosporidiosis in the field (Goodwin 1989, Current 1997) and our experimental findings, support the work of Blagburn and his colleagues, so *C.baileyi* can be added to the list of respiratory pathogens in the poultry industry of Iran. Current and Snyder

(1988) used an IIF test and an ELISA which demonstrated that chickens could generate antibodies to *C.baileyi* following oral inoculation.

To detect antibodies against *C.baileyi* in IIF test, oocysts of *C.parvum* rather than *C.baileyi* were used as the antigen, as they showed considerable antigenic cross reactivity. Hatkin *et al* (1993) developed an indirect ELISA using the antigen obtained from *C.baileyi* oocysts and showed that anti-cryptosporidial serum immunoglobulins (IgM and IgG) were detected as early as 4 days post-inoculation of *C.baileyi* oocysts in broiler chickens.

Diagnosis by faecal examination is not without pitfalls. The oocyst is small (40-70 microns), and may be shed intermittently. In acid fast techniques that are sensitive among various staining methods for *Cryptosporidium* oocysts, the threshold point for positive diagnosis in diarrhea faeces, and normal faeces may be as high as 1×10^4 and 5×10^5 oocysts per gram of faeces, respectively (Fayer 1997, Ungar *et al* 1986). Furthermore, because oocysts appear to be excreted for a relatively short period and clinical disease may be unrecognized in normal hosts and because IgG antibody response appear to persist for at least two months (Fayer 1997), so testing the sera obtained at the end of breeding period (at the slaughter time) can reveal the infection during the life of broiler flocks, and serological evaluation will be useful in establishing more accurate prevalence rates and in describing the epidemiological profile of *Cryptosporidium* infection. Both of the serologic tests used in this study appear to be specific for the detection of antibodies of *C.baileyi* in the sera of chickens, but ELISA is more sensitive, and subjective interpretation in IIF test, limits its practical applicability. The ELISA is a rapid automated and suitable test for screening large number of samples. The result of this study confirms the reports about oocysts shedding in faeces of industrial poultry in Iran. The higher seropositive rate in this study (8.2%) in comparison with positive rates in faecal samples in Iran poultry industry (<3%) (Badiyi 1990, Hoseini-Fard 1999, Moradi 1998, Nikfarjam 1999, Noori *et al* 1994) is due to the short duration of oocysts shedding in faeces, because of self-limiting and rapid disappearing of infection (Fayer 1997) and sensitivity of ELISA.

The higher seropositive rate in broiler flocks of USA (22%-50%) (Snyder *et al* 1988) may be due to using old litter for new flocks in contrast with cleaning and changing of litter for each breeding period in Iran poultry industry.

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