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Diagnosis of bovine cryptosporidiosis by an enzyme-linked immunosorbent assay

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ABSTRACT

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This paper describes an enzyme-linked immunosorbent assay (ELISA) for the diagnosis of cryptosporidiosis. A monoclonal antibody with a high affinity against an oocyst antigen was used to set up the test.

The efficiency of this assay was compared with that of the flotation test; 275 calf faecal samples were examined by the two methods. There was 96% agreement between the two tests. For the 11 conflicting samples, the two tests were repeated and a modified Ziehl–Neelsen staining was performed on faecal smears. All these 11 samples contained few oocysts, but only five and six of them were shown to be positive by the ELISA and flotation tests, respectively. The degree of sensitivity of the ELISA and flotation tests is comparable; samples heavily or moderately contaminated with oocysts are detected by both methods. This ELISA is reliable and never gives rise to false positive results. Nevertheless, as with the flotation test, the occasional case containing very few oocysts will not always be detected by this test. If necessary, very accurate diagnosis can be made by a staining technique or by a direct immunofluorescent assay.

In veterinary medicine, the ELISA seems to be a method of choice; it appears to be a fast and reliable technique which could be used as a routine test for the detection of Cryptosporidium oocysts. Nevertheless the degree of sensitivity must always be borne in mind. There is no need for a microscopic examination, which is an additional advantage.

INTRODUCTION

Cryptosporidium parvum (Phylum, Apicomplexa, Suborder Eimeriina) is an intestinal protozoan parasite which is a well recognized cause of diarrhoeal illness in several mammalian species (Fayer and Ungar, 1986; Pohjola and Lindberg, 1986).

In humans, Cryptosporidium can induce a self-limiting diarrhoea in immunocompetent patients or a severe and prolonged diarrhoea in immunocompromised people (Jokipii and Jokipii, 1986; Soave and Johnson, 1988).

In domesticated animals, cryptosporidiosis is usually a disease of the young.
The economic importance of the disease is a result of its morbidity and the occasional fatal case (Heine and Boch, 1981; Tzipori et al., 1983).

The diagnosis of cryptosporidiosis relies on the identification of oocysts in faecal samples. We describe here an enzyme-linked immunosorbent assay (ELISA) for the detection of parasite antigen in faecal samples. To standardize the test, we used positive and negative faecal samples from naturally infected calves.

MATERIALS AND METHODS

Parasite purification

Calf faeces containing oocysts were stored at 4°C in a 2.5% potassium dichromate aqueous solution.

Purification of the oocysts was achieved as follows. (1) Filtration through several layers of gauze. (2) Dilution of the sample with phosphate-buffered saline (PBS) (NaCl 8 g, KCl 0.2 g, Na₂HPO₄ 2H₂O 1.15 g, KH₂PO₄ 0.2 g pH 7.2)/ether (4 vol/1 vol). Centrifugation at 1500×g for 10 min at room temperature. Discard the supernatant and repeat twice. (3) Wash the pellet twice with PBS. (4) Mix the resulting pellet with Sheather sugar solution (500 g of sucrose in 320 ml H₂O without phenol). (5) The oocysts recovered from the supernatant are washed twice with PBS and (6) incubated for 15 min at room temperature in a solution of detergent (R.B.S. 50, Chemical Product, Belgium)/PBS (1 vol/2 vol). (7) Wash twice with sterile PBS.

Hybridoma production

Balb/c mice were immunized by intraperitoneal injections with at least 10⁷ sonicated oocysts (Ungar et al., 1986). Immunizations were performed on Day 0 (+Freund's complete adjuvant), and on Days 14, 31, 149 and 156 (+Freund's incomplete adjuvant). Four days after the last booster (Day 160), the spleen cells were collected and fused with Sp2-O-Ag14 mouse myeloma cells according to Kohler and Milstein (1975). The supernatant fluid of wells containing hybridoma cells was screened for specific antibodies by either an indirect immunofluorescent antibody test (IFA) using acetone-fixed parasites, as described by Wisher and Rose (1987), or an ELISA using anti-Cryptosporidium rabbit immunoglobulin as capture antibody and sonicated oocysts as antigen. Specific monoclonal antibodies for Cryptosporidium were revealed by a rabbit anti-mouse Ig-horseradish peroxidase (HRP) conjugate (Dakopatts, Glostrup, Denmark).

One hybridoma (S6D12) producing an IgG3, as determined by an anti-mouse Ig isotope ELISA (Amersham, Buckinghamshire, U.K.), was shown to be specific for an oocyst wall antigen both by IFA and ELISA tests. This
monoclonal antibody (Mc Ab) recognized an epitope on fresh oocysts as well as on purified and bleach-treated oocysts. This epitope was not expressed by other parasitic stages (unpublished results).

After cloning by limiting dilution, this hybridoma was injected into pristane-primed Balb/c mice. Ascite fluids were purified by affinity chromatography on a protein A–Sepharose CL-4B column (Pharmacia Fine Chemicals, Uppsala, Sweden) and stored at $-20^\circ \text{C}$.

**Enzyme immunoassay: Development and accuracy**

The S6D12 clone was used to produce the capture antibody and the conjugate for the ELISA.

**Conjugation to HRP**

Conjugation of the purified monoclonal antibody to HRP (P 8375, Sigma, St. Louis) was carried out by the method of Henning and Nielsen (1987). The conjugate was stored at $-20^\circ \text{C}$ with bovine serum albumin (BSA) (100 mg ml$^{-1}$) in 50% glycerol (v/v).

**Coating**

The assay was performed according to the following procedure: 96-well polystyrene immunomodules (Nunc Ref4-68667, Gibco, U.K.) were coated for 18 h at $4^\circ \text{C}$ with 100 µl per well of PBS–thimerosal (1 g l$^{-1}$) containing either 1 µg of Mc Ab anti-Cryptosporidium (uneven rows) or 1 µg of Mc Ab anti-Coronavirus (even rows) as a negative control of non-specific binding of faecal material to the wells. The coating material was then discarded and the wells were saturated for a minimum of 2 h with 200 µl PBS-containing 2% BSA (A-7030 Sigma, St. Louis).

**Sample analysis**

The PBS–BSA solution was discarded and 100 µl of test samples were added to a test and negative control well. Test samples consisted of a small spoonful of calf faeces diluted in 10 ml of PBS–0.1% Tween 20. After 30 min of incubation at room temperature, the modules were thoroughly washed 4–6 times with PBS–0.1% Tween 20 and 100 µl of conjugate S6D12-HRP diluted 100 times in PBS–BSA 2% were added to each well. After 30 min of incubation and an additional four washes, 100 µl of the mixture substrate/chromogen were applied. This mixture consists of 4 mg ortho-phenylenediamine (Ref. 78440 Fluka, Buchs, Switzerland) per 10 ml enzyme substrate buffer (NaH$_2$PO$_4$ 0.0514 M, citric acid 0.0243 M, pH 5) containing 0.03% H$_2$O$_2$. The reaction was stopped with 50 µl of 6 M HCl and the optical density (OD) was measured at 492 nm on a Titertek multiscan reader (Flow Laboratories, Ayrshire, U.K.). On each plate, positive faeces (as determined by flotation)
and a negative control (PBS) were added. The zero was adjusted on the negative control well (coated with Mc Ab anti-Coronavirus) on which the negative control sample (PBS) was applied.

To test the accuracy of the assay, 275 calf faecal samples, taken at random, were tested both by Sheather sugar flotation (Zierdt, 1984) and by ELISA. When a result was doubtful, a faecal smear was stained with the modified Ziehl-Neelsen technique according to Henriksen and Pohlenz (1981). All calf faeces were collected on two farms of the Ardennes area (south of Belgium) with a known history of cryptosporidiosis.

RESULTS

Criteria

The use of a PBS negative control allowed us to detect the non-specific binding of conjugated Mc Ab to the capture Mc Ab. In some cases, there was still a slight signal on this ‘test well’ PBS which ranged, in the results presented here, from 0.015 to 0.053 OD units. These OD values are low, but not negligible, so we decided that to be positive a sample should have a high OD level.

A sample was considered positive when

\[
\text{OD (CR-sample)} - \text{OD (CO-sample)} \geq 0.160
\]

where OD (CR-sample) = optical density on ‘test-well’ coated with Mc Ab anti-Cryptosporidium on which the test sample was applied and OD (CO-sample) = optical density on the ‘negative control well’ coated with Mc Ab anti-Coronavirus on which the test sample was applied.

Accuracy

Two hundred and seventy-five faecal samples, taken at random, were tested to detect the presence of Cryptosporidium oocysts by ELISA and by Sheather sugar flotation. There was good agreement between the two techniques in 264 cases (96%); there were 73 positive samples and 191 negative ones. For each plate, the zero was adjusted on the ‘negative control well’ PBS.

Results of the 73 positive samples

Except for two positive samples that gave high OD values on negative control wells (0.190 and 0.256), the negative control OD values ranged from -0.047 to 0.104. The maximum OD observed on the test well was 1.092 OD units and the maximum OD difference was 1.031. Figure 1 shows the relationship between the OD difference (Y axis) and the flotation results (X axis) for the 73 positive samples. It appears that this relationship cannot be quantified.
Results of the doubtful samples

The 11 doubtful samples are also shown in Fig. 1 (arrows). After the first examination, these 11 samples gave discordant results. They were slightly positive (some oocysts per slide) in the flotation assay and negative in ELISA, or vice versa. The samples were tested again (Flotation 2 and ELISA 2) and in some cases stained by the Ziehl–Neelsen modified technique. The results are summarized in Table 1.

These data indicate that when the faecal sample contains few oocysts both ELISA and flotation assays give variable results. Nevertheless, the ELISA failed to show four samples (8, 9, 10 and 11) that were slightly positive by flotation. On the other hand, Samples 3 and 4 (Table 1), that were positive by ELISA and negative by flotation, are not false-positives. They contained few oocysts, as seen on the stained smear. It is possible that the physical structure of the faecal material (fat, debris, etc.) interfered with the flotation and that very few oocysts reached the top of the tube. The staining technique is always more sensitive, but is time consuming.

Sensitivity

The limit of sensitivity of the ELISA was estimated by serial dilutions of faecal samples; a positive signal was recorded for a well containing > 10 000 oocysts. In some cases, appreciable OD differences (> 0.160) were recorded, whereas the faecal samples were 128 times more diluted than usual. These results indicated that some faecal samples contained > 10^8 oocysts ml⁻¹.
TABLE 1

Samples with conflicting results in the diagnosis of cryptosporidiosis by flotation, ELISA and modified Ziehl-Neelsen staining

| Faecal sample | Flotation 1 | Flotation 2 | ELISA 1 | ELISA 2 | Staining |
|---------------|------------|------------|--------|--------|---------|
| 1             | -          | ≤ 1        | + (0.483) | + (0.356) | n.d.    |
| 2             | -          | ≤ 1        | + (0.186) | − (0.089) | n.d.    |
| 3             | +          | −          | − (0.038) | − (0.059) | +       |
| 4             | +          | n.d.       | − (0.142) | n.d.     | n.d.    |
| 5◊            | -          | −          | + (0.292) | + (0.211) | see 3    |
| 6◊            | -          | −          | + (0.241) | + (0.177) | +       |
| 7             | −          | +          | + (0.353) | n.d.     | n.d.    |
| 8*            | <3         | ≤ 1        | − (0.081) | − (0.087) | n.d.    |
| 9*            | <3         | ≤ 1        | − (0.041) | − (0.050) | n.d.    |
| 10*           | <3         | ≤ 1        | − (0.071) | − (0.081) | +       |
| 11            | <3         | −          | − (0.045) | − (0.052) | see 3    |

Flotation results: − = no demonstrable oocysts; <3 = less than 3 oocysts per microscopic slide; ≤ 1 = no more than 1 oocyst per microscopic field; + = between 1 and 5 oocysts per microscopic field.

ELISA results: − = OD difference < 0.1; + = OD difference ≥ 1; OD differences in parentheses.

Staining results: n.d. = not done; see 3 = 3 oocysts per slide; + = 5 oocysts per slide.

◊ = (Flotation 1 and 2) − and (ELISA 1 and 2) +; * = (Flotation 1 and 2) + and (ELISA 1 and 2) −.

The columns ‘Flotation 2’ and ‘ELISA 2’ recapitulated results obtained after the second trials.

DISCUSSION

This new ELISA has the following features.

1. Specificity. The S6D12 Mc Ab had not been tested against other microorganisms as Garcia et al. (1987) did. During the adaptation of the ELISA we tested ~1000 stools and never encountered false-positive results. Some faecal samples contained a large number of *Eimeria* ssp. oocysts which were not recognized by the S6D12 Mc Ab. In some cases (not encountered in the 275 samples described here), we observed high OD both on the negative control well and on the test well; these signals show non-immunological interaction. In these cases, it is necessary to use another diagnostic method.

2. Rapidity. The 275 faecal samples described in this paper were tested by ELISA in less than one day. Flotation diagnosis of the samples, which implies microscopic observations, took several days.

3. Sensitivity. The sensitivity of this test can be compared with that of the flotation test. When the sample dilution is taken into account, all faecal samples that contain >10^6 oocysts ml^−1 will be identified as positive by the ELISA. In both assays, samples which contain very few oocysts are not always discovered.

When very accurate diagnosis is necessary, we prefer to use a staining technique or a direct immunofluorescent test on a smear sample (Beauvais et al., 1989). This technique, described by Sterling and Arrowood (1986) and by
McLauchlin et al. (1987) gives very good results with the S6D12 Mc Ab conjugated to isofluorothyocyanate. This immunofluorescent test requires microscopic observation, which takes quite a long time. It is for this reason we prefer the ELISA for the routine examination of large samples.

CONCLUSIONS

In veterinary medicine, the detection of very light infections is not always required because many asymptomatic infections have been described (Fayer et al., 1985). When diarrhoea is induced by Cryptosporidium, the infection is usually accompanied by the excretion of millions of oocysts. This assay appears to be as reliable as the classical flotation technique. For large samples, the test is quick and easy to perform. It also appears to be a very useful tool for all persons interested in the epidemiology of the disease.

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REFERENCES

Beauvais, B., Sarfati, C., Debrouin, F., Garth, J.F., Larivière, M. and Deletoille, P., 1989. Evaluation comparative de deux méthodes de dépistage des oocysts de cryptosporidies dans les fèces humaines. Ann. Biol. Clin., 47: 45–46.
Fayer, R. and Ungar, B.L.P., 1986. Cryptosporidium sp and cryptosporidiosis. Microbiol. Rev., 50: 458–483.
Fayer, R., Ernst, J.V., Miller, R.G. and Leek, R.G., 1985. Factors contributing to clinical illness in calves experimentally infected with a bovine isolate of Cryptosporidium. Proc. Helminthol. Soc. Wash., 52: 64–70.
Garcia, L.S., Brewer, T.C. and Bruckner, D.A., 1987. Fluorescence detection of Cryptosporidium oocysts in human fecal specimens by using monoclonal antibodies. J. Clin. Microbiol., 25: 119–121.
Heine, J. and Boch, J., 1981. Kryptosporidien-Infektionen beim Kalb Nachweis, Vorkommen und experimentelle Übertragung. Berl. Muench. Tierarztl. Wochenschrl., 94: 289–292.
Henning, D. and Nielsen, K., 1987. Peroxidase-labelled monoclonal antibodies for use in enzyme immunoassay. J. Immunoassay, 8: 297–308.
Henriksen, S.A. and Pohlenz, J.F.L., 1981. Staining of Cryptosporidia by a modified Ziehl–Neelsen technique. Acta Vet. Scand., 22: 594–596.
Jokipii, L. and Jokipii, A.M.M., 1986. Timing of symptoms and oocyst excretion in human cryptosporidiosis. N. Engl. J. Med., 26: 1643–1647.
Kohler, G. and Milstein, C., 1975. Continuous culture of fused cells secreting antibody of predefined specificity. Nature (London), 256: 495-497.

McLauchlin, J., Casemore, D.P., Harrison, T.G., Gerson, P.J., Samuel, D. and Taylor, A.G., 1987. Identification of Cryptosporidium oocysts by monoclonal antibody. Lancet, 8523: 51.

Pohjola, S. and Lindberg, L.A., 1986. Experimental cryptosporidiosis in mice, calves and chicken. Acta Vet. Scand., 27: 80–90.

Soave, R. and Johnson, W.D., 1988. Cryptosporidium and Isospora belli infections. J. Infect. Dis., 157: 225–229.

Sterling, C.R. and Arrowood, M.J., 1986. Detection of Cryptosporidium sp infections using a direct immunofluorescent assay. Pediatr. Infect. Dis., 5: 139–141.

Sterling, C.R., Seegar, K. and Sinclair, N.A., 1986. Cryptosporidium as a causative agent of traveler’s diarrhea. J. Infect. Dis., 153: 380–381.

Tzipori, S., Smith, M., Halpin, C., Angus, K.W., Sherwood, D. and Campbell, I., 1983. Experimental cryptosporidiosis in calves: clinical manifestations and pathological findings. Vet. Rec., 112: 116–120.

Ungar, B.L.P., Soave, R., Fayer, R. and Nash, T.E., 1986. Enzyme immunoassay detection of immunoglobulin M and G antibodies to Cryptosporidium in immunocompetent and immunocompromised persons. J. Infect. Dis., 153: 570–578.

Wisher, M.H. and Rose, M.E., 1987. Eimeria tenella sporozoites: the method of excystation affects the surface membrane proteins. Parasitology, 95: 479–489.

Zierdt, W.S., 1984. Concentrations and identification of Cryptosporidium sp by use of a parasite concentrator. J. Clin. Microbiol., 20: 860–861.