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Comparison of oocyst shedding and the serum immune response to *Cryptosporidium parvum* in cattle and pigs

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Abstract A comparison was made between oocyst shedding and the presence of specific serum IgG antibodies to *Cryptosporidium parvum* in 108 bovines and 90 pigs. Oocysts were detected by a commercial immunofluorescence assay in feces from 26.8% of bovines and 34.4% of pigs, whereas positive titers as determined by an indirect fluorescent antibody method were found in sera from 12.9% and 48.9% of the respective animals. Infection was significantly most frequent in suckling calves (82.7%) and weaned piglets (87.5%). By contrast, the numbers of seropositives were highest in weaned calves (17.1%) and fattening pigs (76.6%). The results of coprological and serological analysis corresponded in 65.7% of bovines and 56.7% of pigs. When used to diagnose the shedding of cryptosporidial oocysts, the detection of specific IgG antibodies had a sensitivity ranging from 10.3% (cattle) to 58.1% (pigs) and a specificity of 86.1% (cattle) and 55.9% (pigs).

Introduction

Cryptosporidium is a coccidian protozoan parasite found in a wide variety of host species, including mammals, birds, and lower vertebrates (poikilotherms). In mammals, infection usually appears to involve *C. parvum*, a species that infects the epithelial cells of the digestive and respiratory organs. Enteric cryptosporidiosis is being increasingly recognized as a common cause of scours in farm livestock, especially young ruminants (O'Donoghue 1995). In addition to its economic significance, cryptosporidiosis is a zoonosis, since *C. parvum* can be transmitted between host species of mammals. In humans, *C. parvum* is a well-recognized cause of watery diarrhea and vomiting in immunocompetent persons, whereas it causes persistent and severe diarrhea in immunodeficient individuals, especially those with acquired immunodeficiency syndrome (AIDS) (Current and García 1991).

Diagnosis is usually based on identification of *Cryptosporidium* oocysts in fecal smears (Current 1990). However, epidemiology surveys that use coprological techniques may underestimate the prevalence of infections, since oocysts can be excreted sporadically or in low numbers (O'Donoghue 1995). Moreover, conventional concentration and staining procedures have been reported to have a low sensitivity. Although monoclonal antibody-based immunofluorescence assays have been shown to be more sensitive than conventional staining techniques, especially in specimens that contain few parasites and large amounts of debris (Rusnak et al. 1989; García et al. 1992), the threshold of detection of oocysts in stool specimens is high (Weber et al. 1991).

Specific antibodies against oocysts and sporozoites of *Cryptosporidium* have been detected in sera from a wide range of domestic animal species as well as in immunocompetent and immunocompromised persons by fluorescent antibody tests (Tzipori and Campbell 1981; Campbell and Current 1983; Casemore 1987), enzyme-linked immunosorbent assay (ELISA) (Ungar et al. 1986), and Western blotting (Mead et al. 1988). Endogenous stages in histology sections of heavily infected intestinal segments, sporozoites or oocysts were used as the antigen. Serology enables the detection of exposure to *C. parvum*, and prevalence rates recorded in seroepidemiology studies suggest that cryptosporidial infections are more common than those recorded by coprological methods. Ungar et al. (1988) found that more than 50% of people in

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Latin American populations with no history of exposure to infection had specific serum IgG against *Cryptosporidium*.

Using an indirect immunofluorescence procedure, Tzipori and Campbell (1981) reported anti-*Cryptosporidium* antibodies in sera from 100% of cattle and 95% of pigs surveyed, although the serum dilution that these authors considered as positive (1:40) was questioned later (Campbell and Current 1983). Similarly, Vanopdenbosch and Wellemans (1985) detected antibodies to *Cryptosporidium* in sera from 100% of pigs and 92% of cattle, considering as positive immunofluorescent antibody titers of 1:5 or greater. Villacorta et al. (1990) also reported an overall prevalence rate of seropositivity (27.4%) higher than that recorded in coprological analysis (6.3%).

Since limited information is available about the correlation between the shedding of cryptosporidial oocysts and the humoral immune response in animal species more susceptible to cryptosporidiosis, a comparative survey was carried out in fecal and serum samples from cattle and pigs. The objective of the current study was to compare the presence of oocysts in stool samples as determined by a monoclonal antibody-based immunofluorescence reagent and the specific serum IgG antibody levels detected by an indirect fluorescent antibody (IFA) assay using purified oocysts as the antigen.

Materials and methods

Samples

Single fecal and serum samples were individually collected from 108 bovines and 90 pigs selected at random from farms as part of an epidemiology study carried out in Aragón (northeastern Spain). Animals were classified according to age range as shown in Tables 1 and 2. Sera were stored at –20°C until used. Fresh stool specimens were processed by the formalin-ethyl acetate sedimentation technique (Young et al. 1979), and smears of the resulting sediment (25 µl) were examined for the presence of *Cryptosporidium* oocysts using a commercially available monoclonal antibody-based reagent for direct immunofluorescence (Monofluo Kit *Cryptosporidium;* Diagnostics Pasteur, France). Additionally, wet mounts of the concentrate were examined light microscopically for gastrointestinal parasites.

IFA test antigen

Detection of anti-*Cryptosporidium* serum IgG antibodies was performed by an IFA assay. *C. parvum* oocysts purified from feces obtained from naturally infected diarrheic lambs aged 7–15 days were used as the antigen. Stool samples were collected from the rectum using disposable plastic bags and were stored in 5% potassium dichromate (1:1) at 4°C until purification. Oocysts were isolated from the feces using a combination of steps slightly modified from a procedure previously described by Lorenzo-Lorenzo et al. (1993). Briefly, feces were first strained through a series of sieves of 150- and 45-µm mesh size and the suspension recovered was then washed in phosphate-buffered saline (PBS; pH 7.2, 0.04 *M)* by several centrifugation steps (1,250 *g* for 10 min at 4°C) until the potassium dichromate was removed. The sediment was mixed in PBS/ethyl ether (1:1) and centrifuged (800 *g* for 5 min), the supernatant being discarded and this step being repeated until the fecal fat was removed. The sediment was then washed in PBS (1,250 *g* for 10 min) to remove the residual ethyl ether. Oocysts were purified further by isopycnic Percoll-gradient centrifugation. Percoll (Sigma P-1644) solutions with densities of 1.13, 1.09, 1.05, and 1.01 g ml^{-1} were prepared, placed in four 2.5-ml layers in a 15-ml centrifuge tube, overlaid with 1 ml of the sediment with the oocyst suspension, and centrifuged at 650 *g* for 15 min. The band containing purified oocysts, situated between the 1.09- and 1.05-g ml^{-1} layers, was removed and washed three times in PBS (1,250 *g* for 10 min). The oocysts were counted in a hemocytometer and diluted in PBS to a concentration of 15,000–20,000 oocysts/50 µl.

IFA test procedure

For the IFA procedure, immunofluorescence slides were coated with the suspension containing oocysts at the rate of 50 µl/well, fixed with acetone (10 min), and stored at –20°C until used. Fluorescein-labeled rabbit anti-cattle IgG antibody (Nordic Immunological Laboratories, Tilburg, The Netherlands) was used at a 1:40 optimal dilution in 1:10,000 Evans blue. Fluorescein-labeled rabbit anti-pig IgG antibody (Sigma Immunochemicals, St. Louis, Mo., USA) was used at a 1:80 optimal dilution in 1:10,000 Evans blue. Titers of ≥128 in sera from cattle and ≥16 in those from pigs were considered positive. Optimal dilutions and titers regarded as positive were determined by checkerboard titration of fluorescein isothiocyanate (FITC)-conjugated antibodies with positive and negative control sera.

Serial doubling dilutions of test sera samples ranging from 1:16 to 1:256 in PBS were prepared in microtiter plates. Each well of antigen slides was coated with 25 µl of serum dilution and incubated in a moist chamber for 30 min at 37°C. Slides were washed twice for 5 min in PBS and air-dried. Next, 25 µl of anti-species conjugate at the optimal dilution was added to each well and slides were incubated as described above. Slides were washed twice in PBS, air-dried, and mounted in glycerol solution with a cover glass. Microscopical examination was performed at a x400 magnification with a Zeiss epifluorescence microscope. Criteria for positive fluorescence included surface fluorescence of the oocysts; the characteristic suture of cryptosporidial oocysts was seen in most of them. Chi-square and Fisher's exact tests were used for statistical comparisons between the results obtained by coprological versus serological methods.

Results

The distribution of the IFA titers in cattle is shown in Table 1. Positive titers (≥128) were detected in sera from 14 (12.9%) bovines, whereas oocysts of *Cryptosporidium parvum* were found in feces from 29 (26.8%) cattle. Rates of infection, i.e., the percentages of bovines shedding oocysts, were significantly higher in suckling calves aged <1.5 months (82.7%) than in weaned calves aged 1.5–4 months (14.3%) or heifers ranging from 20 to 24

Table 1 Distribution of the IFA titers in cattle

	IFA titer[a]				
Age	<64	64	128	256	Total
<1.5 months	19	8	1	1	29
1.5–4 months	16	13	4	2	35
20–24 months	19	19	6	0	44
Totals	54	40	11	3	108

[a] Titers of ≥ 128 were regarded as positive

Table 2 Distribution of the IFA titers in pigs[a]

Age	IFA titer						
	<16	16	32	64	128	256	Total
15–30 days	14	0	0	0	0	0	14
1–2 months	11	1	3	0	0	1	16
2–6 months	7	12	5	5	1	0	30
Adults	14	16	0	0	0	0	30
Totals	46	29	8	5	1	1	90

[a] Titers of ≥ 16 were regarded as positive

Table 3 Values obtained for some diagnostic parameters of the detection of specific IgG to *Crytosporidium parvum* (IFA procedure) for diagnosis of the shedding of cryptosporidial oocysts

	Cattle	Pigs
Sensitivity	10.3%	58.1%
Specificity	86.1%	55.9%
Positive predictive value	21.4%	40.9%
Negative predictive value	72.3%	71.7%
Global value	65.7%	56.7%

months in age (0) $P<0.001$). By contrast, the percentage of seropositives was higher in weaned calves (17.1%) and heifers (13.6%) than in suckling calves (6.9%), although no statistically significant difference was found. Of the 29 bovines infected by *C. parvum*, only 3 calves aged 7, 15, and 60 days, respectively, had positive titers. The results of coprological and serological analyses (both positive and negative) corresponded in 71 (65.7%) cattle samples. The worst correlation was found in suckling calves (24.1%), whereas the two methods corresponded in 74.3% of weaned calves and 86.4% of heifers.

It is noteworthy that oocysts of *Eimeria* sp. were found in feces from nine bovines aged 1.5–24 months (8.3%). One of them had an IFA titer of 128 for *C. parvum*, but no cryptosporidial oocyst was found in the feces, whereas the remaining eight calves were seronegative for *C. parvum*. No significant difference in the percentage of seropositive samples was found in a comparison of those infected by *C. parvum* (3/29) and those infected by *Eimeria* sp. (1/9).

Table 2 shows the distribution of IFA titers in pigs. A comparative study showed that 44 pigs (48.9%) had positive titers (≥16), whereas oocysts were found in feces from 31 swine (34.4%). The numbers of infected pigs were significantly higher among weaned piglets aged 1–2 months (87.5%) or fattening pigs aged 2–6 months (56.6%) than among suckling piglets aged 15–30 days (0) or adults (0) $P<0.001$). By contrast, the percentage of seropositives was significantly higher in fattening (76.6%) than in weaned (31.2%) or suckling (0) piglets ($P<0.005$), but no statistically significant difference was detected with adults (53.3%).

Only 18 of the 44 seropositive pigs were shedding cryptosporidial oocysts. The results of coprological and serological diagnosis (both positive and negative) corresponded in 55 pigs (56.7%), including 31.2% of weaned piglets, 60% of fattening pigs, and 46.7% of adults. Both methods were negative in 100% of the suckling piglets aged 15–30 days.

No statistically significant difference in the percentage of seropositive samples was found in a comparison of infected versus noninfected animals among both cattle and pigs. The sensitivity, specificity, positive predictive value, negative predictive value, and global value for the detection of specific IgG by the IFA procedure to diagnose fecal oocyst shedding were determined by comparison of the results of serological and coprological analyses (Table 3).

Discussion

On the basis of previous reports showing that the sensitivity of commercially available immunofluorescence assays using monoclonal antibodies is significantly higher than that of acid-fast staining techniques (García et al. 1992; Quílez et al., manuscript submitted for publication), we exclusively used the monoclonal antibody-based method to monitor fecal oocyst shedding. The results of the current study show a poor correlation between shedding of *Cryptosporidium parvum* oocysts and the humoral immune response of specific IgG to *C. parvum*, advising against the use of the IFA serological technique to diagnose the cryptosporidial infection. Lorenzo-Lorenzo et al. (1993) have previously reported that the presence of IgG antibodies in cattle does not necessarily correlate with active infection since oocysts are excreted for a relatively short period.

The specificity of the IFA technique in cattle (86.1%) reflects that the number of false-positive results is low; however, the low sensitivity (10.3%) indicates a high number of false-negative results, most of them being recorded in suckling calves, since only 2 of the 24 infected calves had positive titers for *C. parvum*. These results can be explained by the latent period needed for the antigenic stimulation to induce a humoral response by specific antibodies. The specific serum immune response to enteric cryptosporidiosis in lambs is detected at 7–14 days postinfection (Tzipori and Campbell 1981; Hill et al. 1990). This period ranges from 6–7 days (Williams 1987; Whitmire and Harp 1991) to 14 days (Tzipori and Campbell 1981) to 2–3 weeks postinfection (Harp et al. 1990) in cattle.

On the other hand, the IgG isotype does not seem to be a good indicator of active infection since it rises slowly. In colostrum-deprived lambs, Naciri et al. (1994) found that specific IgM titers were first detected in serum at 4 days post-infection, whereas IgA appeared at 11 days postinfection and specific serum IgG, at 14 days postinfection. Similarly, Hill et al. (1990) and Ortega-Mora et al. (1993) reported that specific serum IgM and IgA were detected in lambs as a peak titer at 15 days postinfection, in association with declining oocyst shed-

ding, whereas levels of specific serum IgG increased slowly and peaked at 30 days postinfection. This could explain the best correlation between stool examination and serodiagnosis reported by Tsaihong and Ma (1990). In determining total immunoglobulins in human sera, these authors found that the IFA technique had a sensitivity of 92.6% and a specificity of 85.8% for the diagnosis of an active infection.

Passive transfer of maternal antibodies has been thought to be an impediment to the immunodiagnosis of cryptosporidiosis, hindering the detection of actively developed antibodies (Ortega-Mora et al. 1993). The half-life of colostral IgG in calves ranges between 10 and 14 days (Levieux 1990), and previous surveys have recorded a high prevalence of antibodies to *Cryptosporidium* in sera from very young calves and lambs using IFA tests (Mann et al. 1987) or ELISA procedures (Peeters et al. 1992; Ortega-Mora et al. 1993; Naciri et al. 1994). However, in the current study, none of the calves aged less than 7 days was seropositive, suggesting that these animals received low levels of colostral antibodies. Although non-hyperimmune colostrum does not protect calves from infection (Harp et al. 1989; Peeters et al. 1992), the scarce maternal transfer inferred in our study may have contributed to the high rate of infection recorded in suckling calves (82.7%).

In pigs the percentage of seropositives (48.9%) was higher than that of infection (34.4%), which agrees with the increase in the sensitivity of the IFA technique (58.1%). Neither oocysts nor positive titers of serum IgG antibodies were detected in suckling piglets, although we cannot rule out the possibility that these animals received antibodies via passive transfer, since all of them were more than 15 days old and the maternal antibody levels drop before that time (Salmon et al. 1990). Indeed, the high percentage of sows found to be seropositive (53.3%) suggests that suckling piglets received a good dose of maternally derived lactogenic immunity, which may protect them up to the age of 12–15 days, after which they usually become inherently resistant (Tzipori et al. 1982), a condition stated by Tzipori (1985) to explain the low prevalence of natural infection in suckling piglets.

Unlike sucklings, weaned calves were more frequently seropositive (17.1%) than infected (14.4%) and oocysts were not found in feces from five of the six seropositive calves. It is likely that these calves had previously been exposed to *C. parvum*, since significant antibody titers have been found to persist longer than oocyst shedding. In cattle with no history of exposure to *C. parvum*, Mosier et al. (1992) showed that antibody responses increased with age, especially between the 1st and 3rd month of age, whereas no significant age-associated change was found in calves older than 3 months. Nevertheless, Harp et al. (1990) demonstrated a 10-fold increase in ELISA titers of anti-*Cryptosporidium* serum antibody in calves raised in isolation from *C. parvum* and challenged for the first time at 3 months of age, concluding that age-related resistance may be ruled out, since 3-month-old calves were as susceptible as 1-week-old calves to infection.

In contrast to infections in ruminants, cryptosporidial infection in pigs is delayed after weaning, and rates were highest in weaned (87.5%) and fattening pigs (56.7%). This agrees with previous studies showing that cryptosporidial infection in swine is especially frequent in recently weaned piglets and in the first stages of fattening (Sanford 1987; Kaminjolo et al. 1993). This could explain why the rates of seropositives were low in weaned piglets (31.2%), probably because most of the pigs were infected but specific antibodies had not yet become detectable. Likewise, the high prevalence of infection found in weaned piglets and the persistence of levels of specific antibodies justify the high percentage of seropositives among fattening pigs (76.7%), which is also indicative of a continuous antigenic stimulation during this period of age, since 56.7% of them were shedding oocysts.

Adults have been thought to be important in the epidemiology of cryptosporidiosis as asymptomatic carriers that intermittently shed oocysts. A high rate of prevalence ranging from 62.4% to 71.7% has occasionally been reported in adult cattle (Lorenzo-Lorenzo et al. 1993; Scott et al. 1995), and infection has also been described in mature pigs (Tacal et al. 1987). In an epidemiology survey previously carried out in Aragón (Quílez et al., unpublished data), we found that 17.8% of cows were infected, whereas none of the sows was shedding *C. parvum* oocysts. In the present study, none of the adults was infected by *C. parvum*, although specific antibodies were detected in both heifers (13.6%) and adult pigs (53.3%), suggesting that these animals had been infected at some time. Lorenzo-Lorenzo et al. (1993) found a seroprevalence of 63.3% (IFA) and 51.1% (ELISA) in adult cattle, although the presence of IgG antibodies to *C. parvum* correlated only with the detection of fecal oocysts in 20.6% of cases. In humans, significant antibody titers have been found to persist for between 1 and 2 years after infection (Ungar et al. 1989). On the other hand, as the parasite is widespread in the environment, it would be reasonable to assume that continued exposure might contribute to the persistence of high levels of serum specific antibodies (Peeters et al. 1992), although reinfection does not always result in a secondary serum antibody response (Harp et al. 1990).

Checks for cross-reactivity with indirect immunofluorescence between *Cryptosporidium* and other sporozoans have shown very little, if any, cross-reactivity with *Toxoplasma gondii*, *Sarcocystis* sp., or *Isospora* sp. (Campbell and Current 1983; Mann et al. 1987; Villacorta et al. 1990; Ortega-Mora et al. 1992). Anusz et al. (1990) did not detect any evidence of cross-reaction between *C. parvum* and four bovine *Eimeria* spp. However, common epitopes to *C. parvum* and several ovine *Eimeria* spp. have been demonstrated, indicating that care must be taken when these methods of serodiagnosis are used because of the high prevalence of *Eimeria* spp. infections in domestic ruminants (Ortega-Mora et al. 1992). Simi-

larly, Lorenzo-Lorenzo et al. (1993) also recorded cross-reactions by IFA and ELISA techniques between *C. parvum* and *Eimeria* spp. from cattle. In the present study, only one of the nine bovines shedding oocysts of *Eimeria* sp. had a positive titer for *C. parvum*, and no statistically significant difference in the percentage of seropositive samples was found in a comparison of those infected by *C. parvum* and those infected by *Eimeria* sp; however, the poor correlation found between the shedding of *Cryptosporidium* oocysts and the titers of specific antibodies makes it difficult for us to reach any conclusion with regard to cross-reactions.

In conclusion, the results of the current study demonstrate that the detection of specific IgG antibodies to *C. parvum* by the IFA technique should not be regarded as being indicative of active infection in cattle and pigs but, rather, should be considered as providing presumptive evidence of previous infections. This serological method appears to have little value for diagnosis in individual cases but would be useful for seroepidemiology studies to determine the prevalence of prior exposure.

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