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Identification of Isolate-Specific Sporozoite Proteins of *Cryptosporidium parvum* by Two-Dimensional Gel Electrophoresis

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Five isolates of *Cryptosporidium parvum* collected from human, horse, and calf sources were compared for differences in sporozoite protein patterns by using two-dimensional gel electrophoresis. Silver-stained two-dimensional gels contained over 300 protein spots from detergent-solubilized sporozoites. A distinguishing 106-kilodalton peptide that shifted in isoelectric point was detected in four of the five isolates. Computerized two-dimensional gel analysis was performed to obtain objective quantitation of the pI shift. Three of these four isolates could be differentiated from one other by the pI shift in this peptide. The fifth isolate was distinguished by the absence of the 106-kilodalton peptide and the presence of a 40-kilodalton peptide that was not observed in any other isolate.

Cryptosporidium parvum is a common enteric protozoal parasite causing diarrheal disease in animals and humans. In immunocompetent individuals the disease is characteristically self-limiting, usually lasting 1 to 3 weeks. Clinical symptoms vary in these individuals from asymptomatic to severe, requiring hospitalization. Immunocompromised individuals (patients undergoing immunosuppressive therapy or patients with acquired immunodeficiency syndrome) often experience chronic infections with life-threatening symptoms (11). The variability of clinical symptoms may be due to host differences or to differences in the virulence of infecting cryptosporidial isolates.

Experimental evidence confirming *C. parvum* isolate variation has been conflicting and limited. Experimentally infected lambs in Scotland exhibited severe symptoms resulting in death (4), whereas similarly infected lambs in Idaho exhibited mild symptoms followed by complete recovery (1). Subclinical infections have been reported in infected cats (13), whereas other reports have mentioned weight loss and persistent diarrhea (7, 18). Swiss mice infected with three isolates of *C. parvum* exhibited no differences in parasite morphology or life cycle features (9). Differences in infectivity and clinical illness have been observed, however, in two isolates infecting calves (11).

More recently, chromosomal DNAs from five isolates of *C. parvum* and one isolate of *Cryptosporidium baileyi* were compared by field-inversion gel electrophoresis (15). This technique showed differences between *Cryptosporidium* species but not among the *C. parvum* isolates. Size-independent differences in the DNA (sequence differences) may remain undetected by the field-inversion gel electrophoresis technique. To investigate this possibility, the current study was initiated to examine pattern variations in detergent-solubilized sporozoite proteins by using two-dimensional gel electrophoresis.

MATERIALS AND METHODS

Oocyst isolates. Five *C. parvum* isolates were obtained for examination. Two isolates originated in calves and were obtained from Harley Moon (National Animal Disease Center, Ames, Iowa), and Philip Klesius (U.S. Department of Agriculture, Auburn, Ala.). The Iowa isolate produced heavy infections in calves, whereas the Alabama isolate was associated with calf morbidity and mortality (11). One isolate originated in horses and was obtained from Thomas Klei (Louisiana State University, Baton Rouge). The Louisiana isolate was associated with morbidity and mortality in foals (8). Two isolates were of human origin: the first was obtained from a traveller returning from Mexico (21), and the second was from a cohort of children living in a Peruvian pueblo hoven (courtesy of Robert Gilman, Johns Hopkins University and Universidad Peruana Cayetano Heredia). The Mexico isolate was obtained from an individual with severe symptoms who required hospitalization, whereas the Peru isolate was pooled from several children who exhibited mild to severe symptoms.

Oocyst production and purification. Oocysts of each isolate of *C. parvum* were used to infect 2-day-old Holstein calves (2×10^8 oocysts per animal). After the onset of oocyst shedding, feces were collected daily, mixed with an equal volume of 5% $K_2Cr_2O_7$, and stored at 4°C. Feces were sieved sequentially through stainless steel screens with a final mesh size of 230 (63-μm pore size). Oocysts were purified by discontinuous sucrose and isopycnic Percoll (Pharmacia Fine Chemicals, Piscataway, N.J.) gradient techniques (6).

Sample preparation. Excystation of oocysts was preceded by hypochlorite treatment to maximize sporozoite yield. Oocysts were suspended in 0.5% hypochlorite (commercial bleach diluted 1:10 in phosphate-buffered saline [PBS]) for 5 min at 4°C and centrifuged. The treated oocyst pellet was suspended in 0.1% sodium thiosulfate (in PBS) to neutralize residual hypochlorite and centrifuged. After two additional PBS washes, oocyst preparations were suspended in PBS containing 0.75% sodium taurocholate and 0.25% trypsin for 1 h at 37°C. Excysted sporozoites (2×10^9) were purified over Percoll gradients and solubilized in 250 μl of NET buffer (2% Nonidet P-40 in 10 mM Tris [pH 7.4] supplemented with 150 mM NaCl, 50 mM phenylmethylsulfonyl fluoride and 50 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone) as previously described (16). Nonexcysted, sucrose gradient-purified oocysts were suspended in NET buffer and

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centrifuged, and the supernatant was collected for analysis. This preparation represented a control for monitoring contaminating proteins (or oocyst surface proteins that might be extracted by detergent). Additionally, an alternate method of solubilization (sonication) was used to detect protein degradation that might have occurred due to enzymatic activity in the excysting solution. Oocysts (2×10^9) suspended in 1.0 ml of PBS with 3 mM EDTA were sonicated for 20 min on an ethanol ice bath with a Branson sonifier (Branson Instruments, Stamford, Conn.), followed by the addition of enzyme inhibitors (50 mM each phenylmethylsulfonyl fluoride and *N*-α-*p*-tosyl-L-lysine chloromethyl ketone).

Two-dimensional gel electrophoresis. Replicate two-dimensional gel separations of all isolate samples were performed as described by O'Farrell (17) and modified by Anderson et al. (2, 3). The first-dimension separation, isoelectric focusing, of the sample was accomplished by using an Iso-20 isoelectric focusing apparatus (Pierce Chemical Co., Rockford, Ill.). Gels were cast with approximately 20 ml of Iso-gel solution (aqueous solution of 4.0% acrylamide, 0.25% bisacrylamide, 2.0% Nonidet P-40, 2.2% ampholytes [pH 3 to 10], 0.034% ammonium persulfate, 0.068% *N,N,N',N'*-tetramethylethylenediamine, 9.9 M urea). Solubilized sporozoite proteins (approximately 15 μg) were mixed 1:3 with sample buffer (4.0% Nonidet P-40, 0.8% ampholytes, 5.0% 2-mercaptoethanol, 9.0 M urea) and loaded onto the top of isoelectric focusing gels. Protein samples used for strain variation studies were first dialyzed against sample buffer in a microdialyzer (Pierce) containing a no. 2 Spectrapor membrane (Spectrum Medical Industries, Los Angeles, Calif.) for 1 h and then mixed with sample buffer. Isoelectric focusing gels were run at a constant 700 V for a total of 12,000 V · h. Isoelectric focusing gels were stored at −70°C or loaded immediately onto the second-dimension acrylamide gel. Second-dimension sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on gradient 10 to 20% polyacrylamide gels. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, application of molecular weight standards, and determination of absolute pI values were performed as previously described (10). Gels were fixed and stained with Gelcode silver stain (19).

Analysis of two-dimensional gels. Silver-stained gels were scanned by the MassComp-based Visage system (BioImage, Ann Arbor, Mich.) and digitized in a 1,024- by 1,024-pixel format. Software supplied by BioImage was used for initial spot detection. Spot lists were created by assignment of *x* and *y* coordinates and integrated intensity values. Additional software, Matchware (Copyright, University of Arizona), was used to match and evaluate gel patterns according to internal standard spots as previously described (10, 20).

The Matchware tolerance settings were assigned at pI 0.22 and 0.80 molecular weight (estimated molecular weight units). The Matchware tolerance settings provide a maximum separation distance permitted between two spots in order for those spots to match.

RESULTS

The sporozoite protein patterns of five *C. parvum* isolates from Iowa, Alabama, Louisiana, Peru, and Mexico were compared. Table 1 shows the geographic origin, host sources, and oocyst production in experimentally infected calves for each isolate. Variations in oocyst production were as great between isolates as they were among replicate calves infected with the same isolate (with the exception of the Louisiana isolate, which yielded the lowest oocyst output).

TABLE 1. *C. parvum* isolate oocyst production in experimentally infected calves

Isolate	Source	Oocysts shed per infected calf[a]
Peru	Human	5.34×10^{10}
Mexico	Human	6.34×10^{10}
Louisiana	Horse	9.97×10^{9}
Alabama	Calf	6.91×10^{10}
Iowa	Calf	6.17×10^{10}

[a] Total number of oocysts shed, days 5 through 9 postinfection.

Excysted sporozoites and sonicated oocyst sample preparations were compared for optimal solubilization and possible degradation. Excysted NET-solubilized preparations did not show observable degradation (e.g., due to enzymatic cleavage) when compared with sonicated preparations. Sonicated preparations did, however, contain greater amounts of insoluble material that caused smearing in the gels. Therefore, excysted sample preparations (solubilized into detergent) were used for all gel comparisons. Sample preparations were judged to be free of contaminating proteins by comparison with gels produced by two-dimensional gel analysis with those from supernatants from detergent-treated intact oocysts.

Two-dimensional gels of sporozoite proteins demonstrated 300 to 400 spots ranging in molecular mass from 150 to 5 kilodaltons (kDa) and ranging in pI from 4 to 8.5. Overall protein patterns of the five isolates were very similar, and a representative gel is shown in Fig. 1. Upon visual comparison, however, one polypeptide of approximately 106 kDa was found to differ in pI between three of the four isolates, and a family of 40-kDa protein spots distinguished the fifth isolate. Figure 2 shows the region of the gel containing the 106-kDa polypeptide (numbers 1, 2, and 3) and the shift that occurred among three of these isolates (Peru, Louisiana, and Iowa isolates). The isoelectric points of the 106-kDa polypeptides for the Louisiana and Alabama (data not shown) isolates were similar (the spots overlapped), and therefore these isolates could not be distinguished.

To objectively quantitate the differences in pI for this peptide, computerized maps of each gel were created with the microcomputer-based Matchware programs. A small

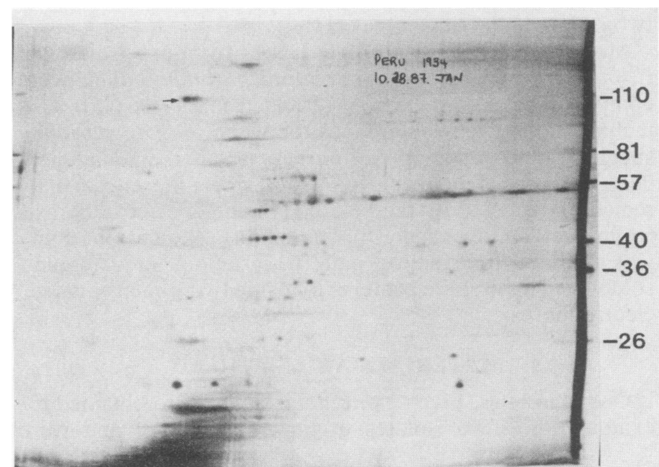

FIG. 1. Typical pattern of sporozoite polypeptides in two-dimensional electrophoresis. Arrow denotes the 106-kDa polypeptide found in the upper left region of the gel.

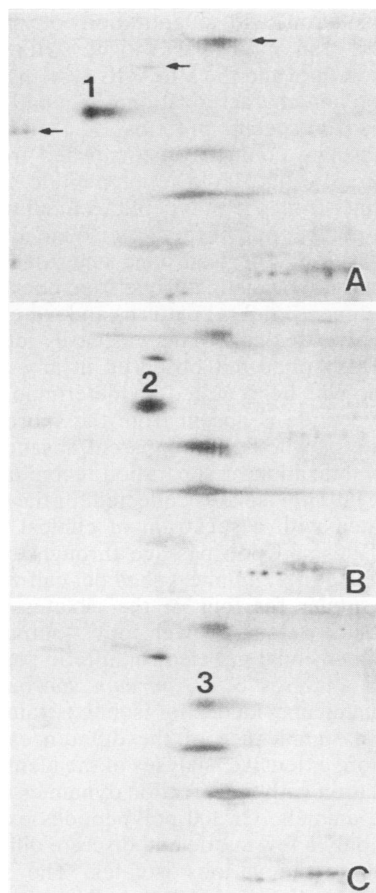

FIG. 2. Region of the gel containing the 106-kDa polypeptide, showing the shift in the 106-kDa polypeptide. Panels A, B, and C represent the Peru, Louisiana, and Iowa isolates, respectively. Spots 1, 2, and 3 indicate the 106-kDa polypeptide. Arrows (panel A) denote the internal standard spots used for computer analyses.

window (120 to 95 kDa and of 5.4 to 6.4 pI) around this peptide was selected for evaluation. This window was matched among the gel patterns of all isolates. Arbitrary pI values of 0, −1, and −2 and estimated molecular weights were defined inside the window by using three internal standards indicated by arrows in Fig. 2A and by numbers 4, 5, and 6 of Fig. 3.

These internal standard spots were selected based on their constant and relative position among all the gels and were used to establish the arbitrary pI scale. The *x* and *y* coordinates of all spots within the window were transformed into arbitrary pI and estimated molecular weight values. After matching, the absolute pI values of the 106-kDa polypeptides were calculated from a standard curve, based on pH measurements from a parallel gel, as previously described (10). The pI and molecular weight values for the 106-kDa polypeptide are listed in Table 2. The Peru and Iowa isolates had discrete spots with average pIs of 5.5 and 6.2 (spots 1 and 3, respectively, of Fig. 3). The Alabama and Louisiana isolates have average pIs of 5.7 and 5.8, which distinguishes them from the Iowa and Peru isolates. The Alabama and Louisiana spots are illustrated as a single spot in Fig. 3.

The Mexico isolate demonstrated a characteristic two-dimensional gel pattern. This isolate could be distinguished from the other isolates by the presence of a prominent family

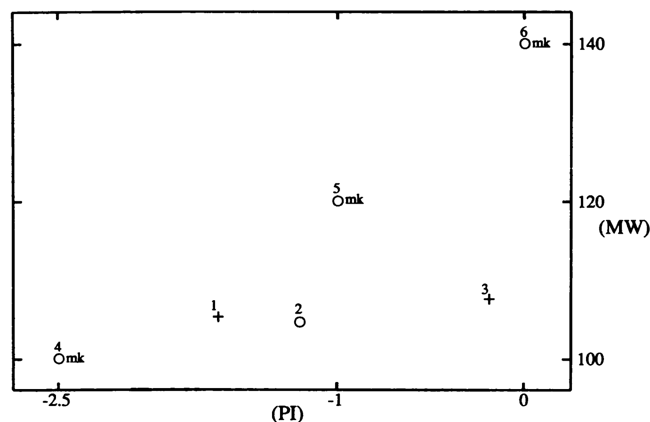

FIG. 3. Computerized map of window surrounding the 106-kDa polypeptide. Arbitrary pI is indicated on the *x* axis, and molecular mass (kDa) is indicated on the *y* axis. Spots 1, 2, and 3 represent the arbitrary pIs of Peru, Louisiana-Alabama, and Iowa isolates, respectively. Spots 4, 5, and 6 represent the internal standard spots with arbitrary pIs of −2.5, −1.0, and 0.0, respectively. The absolute pI of spot 4 is 5.4.

of five spots located at 40 kDa (arrow in Fig. 4B). This family of proteins was not observed in any of the other isolates, and a representative comparison gel is shown in Fig. 4A. The 106-kDa polypeptide of the Mexico isolate was below the level of detection when compared with the other isolates with similar protein loads. However, because the internal markers were also diminished in quantity in both of the Mexico replicate gels, it could not be concluded that the 106-kDa polypeptide is expressed in the Mexico isolate.

DISCUSSION

The issue of isolate variation in *C. parvum* has been difficult to verify biologically, although several case reports suggest this possibility (1, 4, 7, 11, 13, 18). Biochemical evidence for isolate variation has not yet been reported. A recent publication suggests that intact oocysts contain surface proteins that may be useful in differentiating species of cryptosporidia (23). Oocyst surface iodination yielded electrophoretically distinct patterns for three species: *C. parvum*, *C. baileyi*, and *Cryptosporidium muris*. It is not yet clear whether such methods will differentiate cryptosporidial isolates within a given species. Lumb et al. (14) compared five *C. parvum* isolates (four from humans, one from a goat) by using one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis of oocyst extracts; they were unable to distinguish the isolates based on their respective electrophoretic profiles. The present study was initiated to investigate potential biochemical differences among cryp-

TABLE 2. Isoelectric points and molecular masses of *Cryptosporidium* isolate-specific proteins[a]

Isolate	pI			Molecular mass (kDa)		
	Rep 1	Rep 2	Avg	Rep 1	Rep 2	Avg
Peru	5.4	5.5	5.5	106	106	106
Louisiana	5.8	5.7	5.8	106	106	106
Alabama	5.7	5.6	5.7	106	107	106
Iowa	6.1	6.2	6.2	108	107	107

[a] Rep, Replicate.

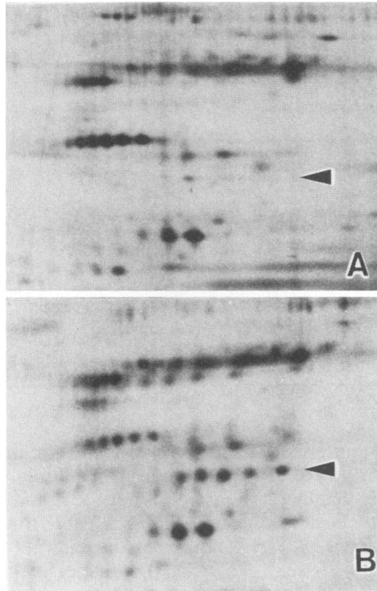

FIG. 4. Region of the gel containing the 40-kDa family of protein spots (arrow) in the Mexico isolate (panel B). Panel A represents the same region of the gel from the Alabama isolate. Note the absence (arrow) of the 40-kDa family of proteins.

tosporidial isolates by using two-dimensional gel electrophoresis.

Two-dimensional gel electrophoresis has been used to detect protein differences in several species of *Eimeria* (22), in schistosomes (5), and in malaria clones (12). These methods were applied to geographically diverse isolates of *C. parvum* that originated in bovine, equine, and human hosts.

Sporozoites were chosen for analysis because they can be isolated in large numbers from oocysts shed by experimentally infected calves (6). In addition, sporozoites are composed of a complex array of proteins (16), which should provide a great deal of information once separated into characteristic patterns by two-dimensional gel electrophoresis.

Silver-stained gels of detergent-solubilized sporozoite proteins showed over 300 polypeptide spots. The overall patterns of the isolate samples displayed significant similarities, suggesting that in general the majority of the constituent proteins are the same between various isolates of the parasite. This observation is consistent with recently published data demonstrating that the two-dimensional protein patterns of related coccidia (*Eimeria* species) are quite similar within isolates of a given species but different between species (22).

Visual comparisons of *C. parvum* isolate sporozoite protein patterns detected differences in a subset of spots located in a narrow region of 120 to 95 kDa and of 5.4 to 6.4 pI. One prominent 106-kDa sporozoite peptide in this region was investigated for its apparent shift in pI among the isolates. Isoelectric point mobility differences in this 106-kDa polypeptide allowed three parasite isolates to be differentiated from one another, whereas two isolates were nearly indistinguishable. The shift in pI for these peptides may reflect protein glycosylation, phosphorylation, acetylation, or amino acid differences (17).

The detailed analysis of two-dimensional gels requires excellent resolution and quantitation of small discrete changes in pI and molecular weight. Utilization of the Matchware software and the VISAGE system allowed accurate resolution and characterization of gel patterns for identification of isolate specific proteins.

The Iowa isolate could be distinguished from the other isolates by the pI of its 106-kDa polypeptide. This polypeptide was distinct from the polypeptides in all other isolates. The 106-kDa polypeptide of the Peru isolate also occupied a distinct pI position. The Louisiana and Alabama isolates, however, could not be distinguished from one another based on their two-dimensional gel patterns and were concluded to be identical. The Mexico isolate was easily identified by an unique 40-kDa peptide not observed in any other isolate. Further work will be required to determine whether the 106-kDa polypeptide is absent from the sporozoites of the Mexico isolate or whether its apparent absence is a consequence of solubilization or separation techniques.

The five *C. parvum* isolates collected for the present study were associated with a spectrum of clinical symptoms in their original hosts. Upon passage through experimentally infected calves, oocyst numbers shed per calf were observed to be quite similar for four of the isolates, whereas the Louisiana isolate yielded a lower oocyst output.

The two-dimensional gel electrophoretic separations and computerized analyses of *C. parvum* sporozoite proteins provide biochemical evidence for isolate (strain) differences. The biological significance of the differences may be addressed by more extensive analyses of the identified proteins and by correlation with the infection dynamics in experimentally infected animals. Of 300 polypeptides examined from each isolate, only a few subtle and discrete differences were observed among the various isolates. The scope of the present study was limited by the small quantities of sporozoite proteins isolated from each isolate and the labor-intensive effort needed for generating samples. Future studies on the biological significance of these specific protein differences should perhaps be limited to a detailed study of two or three isolates.

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