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Genomic Analysis of *Sarcocystis* by Contour-clamped Homogeneous Electric Fields

HUNG-YI SU, ASTRID M. TENTER and ALAN M. JOHNSON

Department of Microbiology and Infectious Diseases, Flinders Medical Centre, Bedford Park, South Australia 5042, Australia

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ABSTRACT

The chromosomal DNA of *Sarcocystis gigantea* and *Sarcocystis muris* were digested with *NotI* and *SfiI* and characterized in contour-clamped homogeneous electric fields. Under these electric fields, the chromosomal DNA of *S. gigantea* and *S. muris* showed different electrophoretic mobilities. Therefore, it appears that this technique may be useful in future studies on the differentiation of *Sarcocystis* species. The results of *NotI* and *SfiI* digestion of *S. muris* chromosomes suggested that there is an absence of a significant number of recognition sites for these two enzymes in the genome of *S. muris*. The addition of the sizes of 15 *SfiI* fragments of *S. gigantea* DNA suggested that the *S. gigantea* genome is larger than 14 mb.

INTRODUCTION

Heteroxenous cyst-forming coccidia of the genus *Sarcocystis* are some of the more prevalent parasites of livestock and also infect many wild mammals, birds, cold blooded animals, and humans (Levine 1986). Little is known about the taxonomy of species placed into the genus *Sarcocystis*. In the past, the taxonomy of *Sarcocystis* species has been based on differences in morphology and life cycle. However, morphological features are often subject to change during the developmental cycle of the parasites, and the complete life cycle of a lot of organisms currently classified as *Sarcocystis* species is still unknown (Levine 1988). In addition, *Sarcocystis* species infecting small mammals and birds often show a low degree of host-specificity (Dubey et al. 1989). These problems have led to a considerable confusion in the description, identification, characterization, and validity of several *Sarcocystis* species.

More recently, isoenzyme electrophoresis proved to be useful for the differentiation of *Sarcocystis* species (Ford et al. 1987). However, a large number of enzyme loci must be examined before valid genetic information can be obtained (Baverstock et al. 1977; Avise and Aquadro 1982).

Pulsed field gel electrophoresis has been used to directly resolve chromosomal DNA in the size range between 200 kb to 2 mb from a variety of parasitic protozoa such as *Leishmania*, *Acanthamoeba*, *Giardia* and *Plasmodium* (Scholler et al. 1986; Rimm et al. 1988; Upcroft et al. 1989; Sheppard et al. 1989). Subsequent studies have developed the techniques to generate and handle DNA in this size range from prokaryotic and eukaryotic organisms, resulting in complete physical maps for large regions of mammalian chromosomes and of the entire *Escherichia coli* chromosome (Smith et al. 1986, 1987a, b; Smith and Cantor 1986, 1987; Lawrance et al. 1987). Thus, it is now possible to analyze megabase DNA molecules from virtually any organism. In this paper we used one version of pulsed field gel electrophoresis, contour-clamped homogenous electric fields (CHEF) electrophoresis (Chu et al. 1986) to characterize two *Sarcocystis* species: *S. gigantea* and *S. muris*.

MATERIALS AND METHODS

Parasites; Samples of *S. gigantea* were obtained from the esophagi of naturally infected sheep slaughtered at a South Australian abattoir. Macroscopically visible *S. gigantea* cysts were excised from the esophageal muscles and opened with a pair of scissors. Released cystozoites were then washed twice in ice-cold phosphate buffered saline. Cystozoites of *S. muris* were isolated from mice infected experimentally with a laboratory strain of *S. muris* as described earlier (Tenter 1987).

Parasite DNA preparation in agarose blocks: Purified parasites were suspended in ice-cold phosphate buffered saline to a density of 5×10^9 cystozoites per ml. The cystozoite suspension was mixed with an equal volume of 2 % low-melting-point agarose (Incert Agarose, FMC Bioproducts, Lot 12447, ME., USA) and then lysed, deproteinated and washed as described by Van der Ploeg et al. (1984).

Restriction enzyme digestion; The agarose blocks containing parasite DNA were digested with one of three restriction enzymes as described by Smith and Cantor (1986). *NotI*, *SfiI* and *EcoRI* were obtained from BRL (MN., USA) and Promega (WI., USA).

DNA size markers; *Saccharomyces cerevisiae*, *Candida albicans* and *Schizosaccharomyces pombe* DNA size markers ranging in size from 260 kb to 1.3 mb, 1.2 mb to 5.0 mb and 3.0 mb to 6.0 mb, respectively, were obtained from Clontech Lab. (CA., USA). Concatemers of bacteriophage lambda DNA cI 8578 ladders ranging in size from 50 kb to 1 mb were purchased from Promega.

CHEF: The CHEF system used here was made by the Department of Biomedical Engineering, Flinders Medical Centre, South Australia according to the configuration described by Chu et al. (1986). The CHEF conditions (electrode angle, voltage, pulse time, agarose concentration and temperature) were varied to give optimum separation of the DNA under analysis.

RESULTS

Comparisons of the electrophoretic mobility of chromosomal DNA from *S. gigantea*, *S. muris*, *S. pombe* and *C. albicans* are shown in Fig. 1. Because of the great differences in size of the markers and the *Sarcocystis* DNA, it was not possible to find one set of CHEF conditions which separated the chromosomes in the two *Sarcocystis* species and the two markers. However, at conditions of 30 V, 60 min pulse time, 0.6 % agarose and 9 ^{O}C, most of the *S. gigantea* chromosomal DNA was in a similar size range to *S. pombe* while most of the *S. muris* chromosomal DNA was smaller than the *C. albicans* chromosomal DNA.

S. gigantea chromosomal DNA was cut with different amounts of *NotI* as shown in Fig. 2. Complete digestion of *S. gigantea* DNA was accomplished by 5 U or more of *Not*I, and gave 4 bands which were about 700 kb, 600 kb, 500 kb and 460 kb, and a smear of DNA from 400 kb to less than 50 kb, using a 2.5 min pulse time. Complete *NotI* digestion of *S. gigantea* DNA at 1.0 min pulse time gave 7 bands at about 680 kb, 580 kb, 550 kb, 530 kb, 475 kb, 460 kb and 440 kb, a DNA smear from about 380 kb to less than 50 kb and a bulk of DNA from 200 kb to 50 kb as shown in Fig. 3. Complete digestion of *S. gigantea* DNA at 1.0 min pulse time was achieved with 5 U or more of *SfiI* (Fig. 4). *SfiI* digestion of *S. gigantea* DNA at 2.5 min pulse time gave 14 bands at about 1.3 mb, 1.28 mb, 1.265 mb, 1.24 mb, 1.1 mb, 1.05 mb, 1.01 mb, 950 kb, 920 kb, 870 kb, 820 kb, 770 kb, 700 kb and 600 kb, and a smear of DNA from about 430 kb to less than 50 kb as shown in Fig. 5.

S. muris DNA was not cut by using different amounts of *NotI* and *SfiI* since the same electrophoretic pattern of DNA was observed at 0, 0.5, 5, 15 and 30 U of the enzymes (data not shown). *EcoRI* digestion of *S. muris* chromosomal DNA gave a smear of DNA less than 50 kb as shown in Fig. 6.

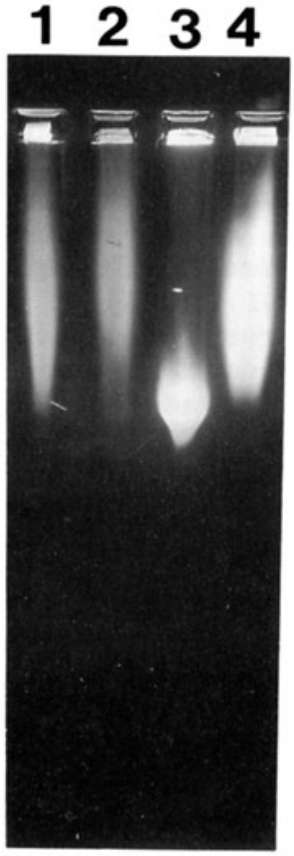

Fig. I. Comparison of electrophoretic mobility of chromosomal DNA of two *Sarcocystis* species and two molecular size markers at 30 V, 120 min pulse time, 0.6 % agarose and 9 ^{O}C. Lane 1, *S. pombe*; lane 2, *S. gigantea*; lane 3, *S. muris*; lane 4, *C. albicans*.

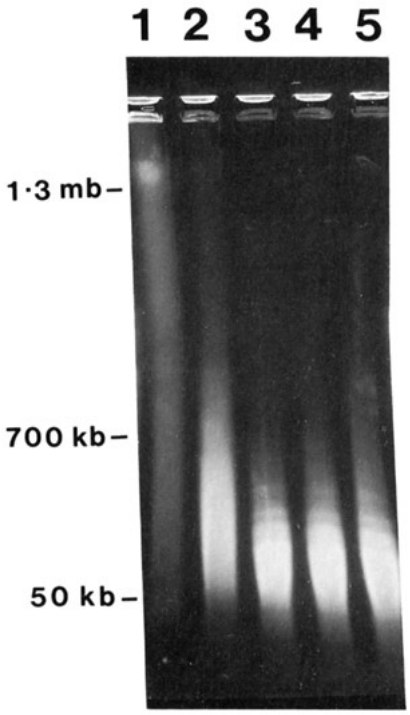

Fig. II. *Not*I digestion of *S. gigantea* chromosomal DNA at 120 V, 2.5 min pulse time, 1 % agarose and 7^{O}C. Lane 1, 0 U; lane 2, 0.5 U, lane 3, 5 U; lane 4, 15 U, lane 5, 30 U *Not*I.

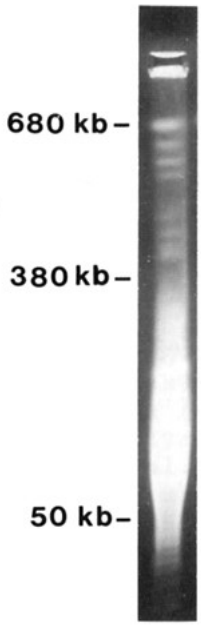

Fig. III. *Not*I digestion of *S. gigantea* chromosomal DNA at 120 V, 1 min pulse time, 1 % agarose and 7 ^{O}C.

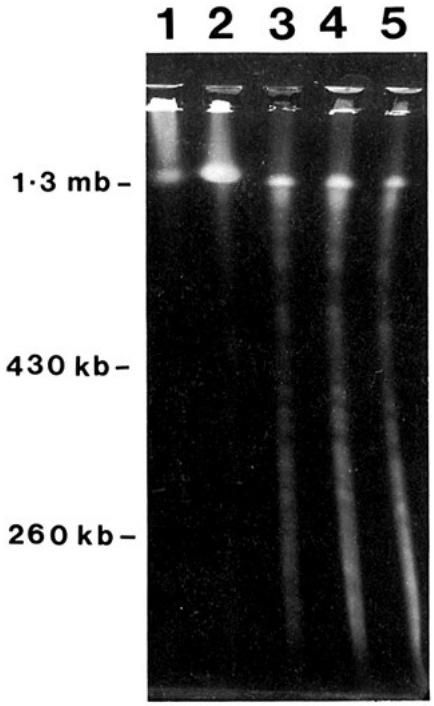

Fig. IV. *Sfi*I digestion of *S. gigantea* chromosomal DNA at 120 V, 1 min pulse time, 1 % agarose and 7^{O}C. Lane 1, 0 U; lane 2, 0.5 U; lane 3, 5 U; lane 4, 15 U; lane 5, 30 U *Sfi*I.

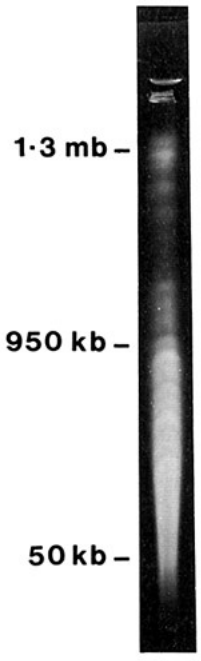

Fig. V. *Sfi*I digestion of *S. gigantea* chromosomal DNA at 120 V, 2.5 min pulse time, 1 % agarose and 7 ^{O}C.

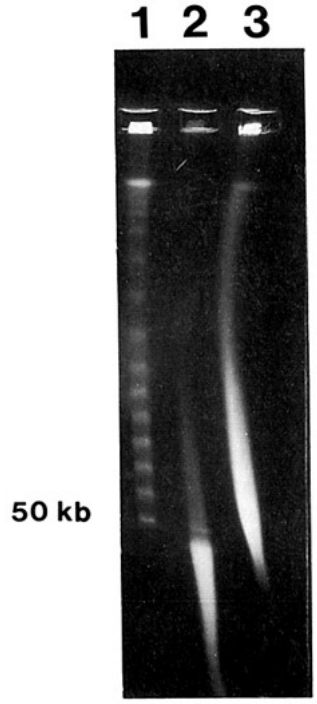

Fig. VI. *Eco*RI digestion of *S. muris* chromosomal DNA at 120 V, 1 min pulse time, 1 % agarose and 7 ^{O}C. Lane 1, bacteriophage lambda ladders; lane 2, *Eco*RI digested *S. muris* DNA; lane 3, undigested *S. muris* DNA.

DISCUSSION

The taxonomy of *Sarcocystis* species has been based on differences in morphology and life cycle, and the use of isoenzyme electrophoresis. Recently, phylogenetic relationships of the apicomplexan protist *Sarcocystis* have been determined by the comparison of partial sequences of small subunit ribosomal RNA (Johnson et al. 1988; Barta et al. 1991; Gajadhar et al. 1991; Tenter et al. 1992). Here we used CHEF to compare the genomes of two *Sarcocystis* species.

*Not*I recognizes and cuts the sequence GC/GGCCGC and *Sfi*I recognizes and cuts the sequence GGCCNNNN/NGGCC. These two enzymes cut mammalian DNA into average sizes of 1000 kb to 1,500 kb, and 200 kb, respectively. Although the fragment sizes obviously depend on the G/C content of the genome under investigation, or the presence of highly reiterated G/C-rich regions, we believed that these enzymes may be useful for analysis of the *Sarcocystis* genome. They had recently been used to analyze polymorphisms in *Theileria*, another apicomplexan genus (Morzaria et al. 1990). Using these infrequently cutting enzymes, *S. gigantea* DNA was digested into 4 to 7 fragments from 440 kb to 700 kb by *Not*I and 14 to 17 fragments from 150 kb to 1.3 mb by *Sfi*I. *Not*I digestion of *S. gigantea* DNA at 2.5 min and 1.0 min pulse times produced a DNA smear from about 430 kb to 50 kb and further studies using pulse times shorter than 1.0 min may be required to resolve the unseparated species in the smear. *Sfi*I digestion of *S. gigantea* DNA at 2.5 min and 1.0 min pulse times produced a DNA smear in the lower size range and a DNA bulk in the higher size range, respectively. To resolve these DNA smear and DNA bulk, pulse times between 1.0 and 2.5 min may be required.

The genome size of *S. gigantea* was estimated to be larger than 14 mb by the addition of the sizes of the *Sfi*I fragments. However, this value is likely to be an underestimate since the DNA smear from 50 kb to 430 kb containing unseparated species has not been included into the size calculation. The absolute DNA content of *S. muris* cystozoites has been found to be 227 x 10^{-15} g (Mackenstedt et al. 1990), while the value for *Sarcocystis cruzi* cystozoites has been found to be 216 x 10^{-15} g (Cornelissen et al. 1984). Mackenstedt et al. (1990) suggested that these differences are species-specific.

S. muris DNA was not cut by *Not*I and *Sfi*I. This could be due to the presence of inhibitors in the agarose block preparations, or the absence of a large number of sites in the *S. muris* genome for these enzymes. However, *S. muris* DNA was cut by *Eco*RI. This suggests that the lack of cutting by *Not*I and *Sfi*I is not due to inhibitors, but is probably due to the presence of few or no recognition sites for *Not*I and *Sfi*I in the genome of *S. muris*. In addition, the lack of *Not*I and *Sfi*I cutting of *S. muris* is not likely to be due to technical parameters (Smith et al. 1988), because although *Sarcocystis* empirically appears to have an increased protein content in comparison with other apicomplexans we have investigated, the blocks were treated with proteinase K and washed extensively before restriction enzyme cutting. *Not*I and *Sfi*I restriction fragment length polymorphisms between *S. gigantea* and *S. muris* could not be obtained in this study since *S. muris* DNA was not cut by these two enzymes and thus differentiation of these two species based on *Not*I and *Sfi*I restriction fragment length polymorphisms was not possible.

Interestingly, Morzaria et al. (1990) found that they could not compare the *Not*I and *Sfi*I restriction fragment length polymorphism patterns of *Theileria parva* and *Theileria mutans*. However, in this case it was due to the fact that *Not*I and *Sfi*I both cut the genome of *T. mutans* much more frequently than the genome of *T. parva*, with the majority of fragments being less than 65 kb. Here, as in our work on *Sarcocystis*, G/C content and/or reiteration of G/C-rich regions in the genome of two species in the one genus appears to vary greatly.

The other point raised by Morzaria et al. (1990), which also applies equally to the work described here, is the possibility of heterogeneity among the parasites obtained from animals naturally infected in the field. This question can only be answered by the use of cloned parasite stocks, and due to the long incubation time of most *Sarcocystis* species, this is likely to be very difficult to do.

Because the sizes of *S. muris* and *S. gigantea* and the two size markers *S. pombe* and *C. albicans* differed so greatly, we were unable to find one set of CHEF conditions that allowed adequate separation of them all. However, the different electrophoretic mobilities of *S. muris* and *S. gigantea* DNA in the CHEF did appear to differentiate the two species.

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REFERENCES

Avise, J. C. & Aquadro, C. F. 1982. A comparative summary of genetic distances in the vertebrates: Patterns and correlations. *Evol. Biol. 15*: 151-185.

Barta, J. R., Jenkins, M. C. & Danforth, H. D. 1991. Evolutionary relationships of avian *Eimeria* species among other apicomplexan protozoa: Monophyly of the Apicomplexa is supported. *Mol. Biol. Evol. 8*: 345-355.

Baverstock, P. R., Watts, C. H. S. & Cole, S. R. 1977. Electrophoretic comparisons between allopatric populations of five Australian pseudomyine rodents (Muridae). *Aust. J. Biol. Sci. 20*: 471-485.

Chu, G., Vollrath, D. & Davis, R. W. 1986. Separation of large DNA molecules by contour-clamped homogenous electric fields. *Science 234*; 1582-1585.

Cornelissen, A. W. C. A., Overdulve, J. P. & Van der Ploeg, M. 1984. Determination of nuclear DNA of

five eucoccidian parasites, *Isospora (Toxoplasma) gondii*, *Sarcocystis cruzi*, *Elmeria tenella*, *E. acervulina* and *Plasmodium berghei*, with special reference to gamontogenesis and meiosis in *I. (T.) gondii*. *Parasitology 88*: 531-553.

Dubey, J. P., Speer, C. A. & Fayer, R. 1989. Sarcocystosis of animals and man. CRC Press, Boca Raton.

Ford, G. E., Fayer, R., Adams, M., O'Donoghue, P. J., Dubey, J. P. & Baverstock, P. R. 1987. Genetic characterization by isoenzyme markers of North American and Australasian isolates of species of *Sarcocystis* (Protozoa, Apicomplexa) from mice, sheep, goats and cattle. *Syst. Parasitol. 9*: 163-167.

Gajadhar, A. A., Marquardt, W. C., Hall, W. C., Gunderson, J., Ariztia-Carmona, E. V. & Sogin, M. L. 1991. Evolutionary relationships among apicomplexans, dinoflagellates, and ciliates: Ribosomal RNA sequences of *Sarcocystis muris*, *Theileria annulata*, and *Crypthecodinium cohnii*. Mol. *Biochem. Parasitol. 45*: 147-154.

Johnson, A. M., Illana, S., Hakendorf, P. & Baverstock, P. R. 1988. Phylogenetic relationships of the apicomplexan protist *Sarcocystis* as determined by small subunit ribosomal RNA comparison. *J. Parasitol. 74*: 847-860.

Lawrance, S. K., Smith, C. L., Weissmann, S. M. & Cantor, C. R. 1987. Megabase-scale mapping of the HLA gene complex by pulsed field gel electrophoresis. *Science 235*: 1387-1390.

Levine, N. D. 1986. The taxonomy of *Sarcocystis* (Protozoa, Apicomplexa) species. *J. Parasitol. 72*: 372-383.

Levine, N. D. 1988. The protozoan phylum Apicomplexa, vol. 2, CRC Press, Boca Raton.

Mackenstedt, U., Wagner, D., Heydorn, A. O. & Mehlhorn, H. 1990. DNA measurement and ploidy determination of different stages in the life cycle of *Sarcocystis muris*. *Parasitol. Res. 76*: 662-668.

Morzaria, S. P., Spooner, P. R., Bishop, R. P., Musoke, A. J. & Young, J. R. 1990. *Sfi*I and *Not*I polymorphisms in *Theileria* stocks detected by pulsed field gel electrophoresis. *Mol. Biochem. Parasitol. 40*: 203-212.

Rimm, D. L., Pollard, T. D. & Hielter, P. 1988. Resolution of *Acanthamoeba castellanii* chromosomes by pulsed field gel electrophoresis and construction of the initial linkage map. *Chromosoma 97*: 219-223.

Scholler, J. K., Reed, G. D. & Stuart, K. 1986. Molecular karyotype of species and subspecies of *Leishmania*. *Mol. Biochem. Parasitol. 20*: 279-293.

Sheppard, M. Thompson, J. K., Anders, R. F., Kemp, P. J. & Lew, A. M. 1989. Molecular karyotyping of the rodent malarias *Plasmodium chabaudi*, *Plasmodium berghei* and *Plasmodium vinckei*. *Mol. Biochem. Parasitol. 34*: 45-52.

Smith, C. L. & Cantor, C. R. 1986. Approaches to physical mapping of the human genome. *Cold Spring Harbour Symp. Quant. Biol. 51*: 115-122.

Smith, C. L. & Cantor, C. R. 1987. Purification, specific fragmentation and separation of large DNA molecules. *Meth. Enzymol. 155*: 449-467.

Smith, C. L., Warburton, P. E., Gaal, A. & Cantor, C. R. 1986. Analysis of genome organization and rearrangements by pulsed field gradient gel electrophoresis. *Genet. Eng. 8*: 45-70.

Smith, C. L., Econome, J. G., Schutt, A., Klco, S. & Cantor, C. R. 1987a. A physical map of *E. coli* K12 genome. *Science 236*: 1448-1453.

Smith, C. L., Lawrance, S. K., Gillespie, G. A., Cantor, C. R. Weissmann, S. M. & Collins, F. S. 1987b. Strategies of mapping and cloning macroregions of mammalian genomes. *Meth. Enzymol. 151*: 461-489.

Tenter, A. M. 1987. Comparison of enzyme-linked immunosorbent assay and indirect fluorescent antibody test for the detection of IgG antibodies to *Sarcocystis muris*. *Zbl. Bakteriol. Hyg., Series A 267*: 259-271.

Tenter, A. M., Baverstock, P. R. & Johnson, A. M. 1992. *Sarcocystis* Lankester, 1882; Phylogenetic relationships of species from *Ovies aries*, *Capra hircus*, *Bos taurus*, and *Mus musculus* based on ribosomal RNA sequence comparison. Submitted.

Upcroft, J. A., Boreham, P. F. L. & Upcroft, P. 1989. Geographic variation in *Giardia* karyotypes. *Int. J. Parasitol. 19*: 519-527.
Van der Ploeg, L. H. T., Schwartz, D. C., Cantor, C. R. & Borst, P. 1984. Antigenic variation in *Trypanosoma brucei* analyzed by electrophoretic separation of chromosome-sized DNA molecules. *Cell 37*: 77-84.

Address corespondence and reprint requests to Dr. Alan M. Johnson, Department of Microbiology, School of Biological and Biomedical Sciences, University of Technology Sydney, Westbourne Street, Gore Hill, NSW, Australia 2065.