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Antibody to a Recombinant Merozoite Protein Epitope Identifies Horses Infected with *Babesia equi*

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Horses infected with *Babesia equi* were previously identified by the presence of antibodies reactive with a merozoite surface protein epitope (D. P. Knowles, Jr., L. E. Perryman, L. S. Kappmeyer, and S. G. Hennager. J. Clin. Microbiol. 29:2056–2058, 1991). The antibodies were detected in a competitive inhibition enzyme-linked immunosorbent assay (CI ELISA) by using monoclonal antibody 36/133.97, which defines a protein epitope on the merozoite surface. The gene encoding this *B. equi* merozoite epitope was cloned and expressed in *Escherichia coli*. The recombinant merozoite protein, designated equi merozoite antigen 1 (EMA-1), was evaluated in the CI ELISA. Recombinant EMA-1 bound antibody from the sera of *B. equi*-infected horses from 18 countries. The antibody response to EMA-1 was then measured in horses experimentally infected with *B. equi* via transmission by the tick vector *Boophilus microplus* or by intravenous inoculation. Anti-EMA-1 antibody was detected 7 weeks post-tick exposure and remained, without reexposure to *B. equi*, for the 33 weeks of the evaluation period. The data indicate that recombinant EMA-1 can be used in the CI ELISA to detect horses infected with *B. equi*.

The tick-borne hemoprotozoan *Babesia equi* causes disease that affects horses worldwide (4, 14, 17). Infection followed by fever, anemia, and icterus can occur when uninfected horses are moved into endemic areas or infected horses are moved into nonendemic areas in which an appropriate tick vector is present (13). Horses that survive the initial infection are lifelong carriers of *B. equi* (10). Prevention or clearance of *B. equi* infection by vaccination or drug therapy is not currently possible.

Many countries, including the United States, do not allow importation of *B. equi*-infected horses (4). Since 1969, the U.S. Department of Agriculture has used the complement fixation test (CFT) (2, 3, 9) as the official assay for detecting anti-*B. equi* antibody. The limitations associated with CFT, including the inability to evaluate sera with anticomplement activity, have been described (12, 15). Also, since it has not been possible to continuously cultivate *B. equi* in vitro, antigen for CFT must be produced by the infection of splenectomized horses.

A monoclonal antibody (MAb)-based competitive inhibition enzyme-linked immunosorbent assay (CI ELISA), which overcomes the limitations associated with CFT, has been described (12). That assay was shown to be specific for anti-*B. equi* antibody in that sera from horses infected with *Babesia caballi* were negative in the CI ELISA (12). The CI ELISA uses immunoglobulin G1 (IgG1) MAb 36/133.97, which binds to a peptide epitope on the surface of *B. equi* merozoites (11), and merozoite antigen obtained from splenectomized horses infected with *B. equi*. A goal of the present study was to produce a molecular clone that expresses the *B. equi* merozoite protein bound by MAb 36/133.97. This *B. equi* recombinant merozoite protein, designated equi merozoite antigen 1 (EMA-1), was evaluated for its ability to bind antibody from the sera of *B. equi*-infected horses from 18 countries. In addition, the CI ELISA with recombinant EMA-1 was used to measure the development of specific antibody response in horses infected with *B. equi* via transmission by the tick vector *Boophilus microplus* (19). The data presented here demonstrate that the CI ELISA with recombinant EMA-1 can be used to detect anti-*B. equi* antibody in the sera of carrier horses. Also, the data show that an anti-EMA-1 antibody response is maintained in sera of carrier horses without reexposure to *B. equi*.

MATERIALS AND METHODS

***B. equi* strain and anti-*B. equi* immune sera.** The *B. equi* strain used was obtained in 1976 from a horse in Florida and was described previously (11). A nonsplenectomized horse (horse H5) was infected by two intravenous injections spaced 2 months apart. Initial inoculation of H5 was with 30 ml of a Florida *B. equi* first-passage stabilate containing 5.6×10^6 viable organisms per ml (11). The second inoculation was with a 2.0-ml blood stabilate in which 49% of erythrocytes were parasitized. The stabilate was prepared as described previously (16). The anti-*B. equi* immune sera derived from horses infected via experimental tick transmission are described below. The six equine serum samples used as controls in the CI ELISA were from a breeding herd maintained at Washington State University, Pullman.

One hundred twenty-three equine serum samples previously tested for antibodies to *B. equi* by CFT (2, 3, 9) were obtained from the National Veterinary Services Laboratories, U.S. Department of Agriculture, Ames, Iowa. Eighteen of these serum samples were from different countries and were previously shown to be positive for anti-*B. equi* antibody by CFT and CI ELISA with native antigen (12). The

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additional 105 equine serum samples from Brazil, Chile, Colombia, Dominican Republic, Ecuador, Poland, Spain, and Venezuela had not been tested by CI ELISA.

***B. equi* cDNA expression library.** Poly(A)$^+$ RNA was isolated by poly(U)-Sephadex (Bethesda Research Laboratories, Gaithersburg, Md.) chromatography from erythrocytes infected with *B. equi* merozoites as described previously (11). Purified poly(A)$^+$ RNA was used to prepare an erythrocyte-stage cDNA library in lambda ZAP II (Stratagene, La Jolla, Calif.) by a method modified from that of Gubler and Hoffman (7) by using *Eco*RI adapters (Pharmacia LKB, Piscataway, N.J.). The cloned insert in plaque-purified lambda phage was transferred into the Bluescript SK phagemid by using the in vivo excision capabilities of lambda ZAP II (18).

Immunoscreening. Screening was done with MAb 36/133.97, which binds to a conserved merozoite surface protein epitope on *B. equi* (11). Plaque lifts on isopropyl-β-D-thiogalactopyranoside-soaked nitrocellulose were screened by using MAb 36/133.97 (2 μg/ml); this was followed by screening with rabbit anti-murine antibody and ^{125}I-labeled protein A. Positive plaques were identified following autoradiography (22). A recombinant phagemid excised from positive, plaque-purified lambda phage was tested for expression by a similar method by using colony lifts from transformed, ampicillin-resistant *Escherichia coli* (XL1-Blue strain) (22). The excised phagemid was designated pEma1.

Production of recombinant EMA-1. *E. coli* DH5 (Bethesda Research Laboratories) was transformed (8) with 1 μg of either pBluescript or pEma1. The entire transformation reaction was added to 250 ml of YT broth containing 12.5 mg of ampicillin and 1 mM isopropyl-β-D-thiogalactopyranoside, and the mixture was incubated overnight at 37°C and 250 rpm. Bacteria were pelleted by centrifugation at 1,000 × *g* for 10 min at 4°C. The pellet was resuspended in 40 ml of proteinase inhibition buffer (50 mM Tris [pH 8.0], 5 mM EDTA, 5 mM iodoacetamide, 0.1 mM Na-*p*-tosyl-L-lysine chloromethyl ketone, and 1 mM phenylmethylsulfonyl fluoride) and repelleted. The pellet was then resuspended in 20 ml of proteinase inhibition buffer containing 1 mg of lysozyme per ml and was incubated on ice for 20 min. Nonidet P-40 was added to 1%; the lysate was vortexed briefly and incubated on ice for 10 min. The lysate was then sonicated twice for 20 s each time at 100 W. The bacterial lysate was centrifuged at 12,000 × *g* for 10 min at 4°C, and the supernatant, containing recombinant EMA-1, was stored at 4°C. The protein concentrations of the supernatants were determined by the DC Protein Assay (Bio-Rad Laboratories, Richmond, Calif.).

Immunoblotting. Lysates of bacteria transformed with either pEma1 or pBluescript were boiled for 3 min in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer (final concentrations, 25 mM Tris [pH 6.8], 2% [wt/vol] SDS, 15% [vol/vol] glycerol, 2.5% 2-mercaptoethanol, and a few crystals of bromophenol blue) and were electrophoresed in an SDS–7.5 to 17.5% polyacrylamide gradient slab gel with a 5% stacking gel (20). Transfer to nitrocellulose was performed by standard techniques (21). Membranes were blocked in Tris–Tween 20 buffer containing 5% milk. Recombinant EMA-1 was detected with MAb 36/133.97 (2 μg/ml); this was followed by treatment with peroxidase-conjugated donkey anti-murine IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, Pa.) and enhanced chemiluminescence (Amersham International plc, Amersham, United Kingdom). The control consisted of an irrelevant IgG1 isotype MAb.

Transmission of *B. equi* via *B. microplus*. Transmission of *B. equi* via the tick vector *B. microplus* was performed as described previously (19). Approximately 10,000 (0.5 g) unfed *B. microplus* larvae (normal colony) were applied under a cloth patch to a pony with an intact spleen 3 days after intravenous inoculation of 25 ml of blood stabilate of a Florida *B. equi* isolate mixed with an equal volume of 10% normal heterologous equine serum in phosphate-buffered saline (PBS). Ticks were removed as replete nymphs 13 days after application and were held for molt off the host for 6 days. On day 6, male and female ticks were applied to horses H16 and H20, which had intact spleens, and were allowed to feed for 8 days. Sera for testing in the CI ELISA were collected weekly.

CI ELISA with recombinant EMA-1. The CI ELISA was performed as described previously (12), with the exception that recombinant EMA-1 was used as the source of antigen. Briefly, 0.2 μg of bacterial lysate containing recombinant EMA-1 in PBS (pH 7.4) with 20 mM $MgCl_2$ was coated onto individual wells of flat-bottom plates (Immunlon 2; Dynatech Laboratories, Chantilly, Va.). All incubations were done at room temperature. Coated wells were incubated for 16 h and blocked for 2 h with buffer A (PBS with 0.2% Tween 20 and 20% milk). Equine sera diluted 1:10 in buffer A were added. Following a 30-min incubation, 0.125 μg of MAb 36/133.97 in buffer A was added. A 1-h incubation was followed by washing three times with buffer B (PBS with 0.2% Tween 20). Biotinylated equine anti-murine IgG in buffer A was added and incubated for 30 min; wells were washed three times with buffer B. The addition of avidin-conjugated alkaline phosphatase in buffer B (Vector Laboratories, Burlingame, Calif.) was followed by a 30-min incubation. Wells were washed three times with buffer B, and 100 μl of 1.0 μg of *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, Mo.) per μl in 100 mM $NaHCO_3$ (pH 9.5) with 10 mM $MgCl_2$ was added to each well. Following a 30-min incubation, reactions were stopped with 50 μl of 0.2 M EDTA and the optical density at 410 nm was read on a Dynatech MR-5000 ELISA plate reader.

The six equine control serum samples were tested at a 1:10 dilution in duplicate each day. Means and standard deviations of the optical density for the control sera were calculated following each test day. A serum sample was considered positive for antibody to *B. equi* if it inhibited the binding of MAb 36/133.97 such that the mean duplicate optical density for that dilution of test serum sample was at least 3 standard deviations below the mean optical density of the control serum sample for that test day. Sera yielding discrepant results were retested in the CI ELISA at a 1:2 dilution.

CFT. The CFT was performed at the National Veterinary Services Laboratories, Veterinary Services, Animal and Plant Health Inspection Service, U.S. Department of Agriculture, by previously described methods (2, 3, 9).

RESULTS

Expression of recombinant EMA-1. Figure 1 shows that pEma1 predominantly expressed a 43-kDa protein in *E. coli* DH5 (Bethesda Research Laboratories). The numerous smaller proteins were most likely the result of limited degradation (5, 6). In the immunoblot assay, MAb 36/133.97 binds native merozoite proteins of 28 and 34 to 36 kDa (11). Expression of pEma1 in the Bluescript SK phagemid should result in a fusion product that contains 4.5 kDa of the β-galactosidase protein. Therefore, the approximate predicted molecular mass range of a full-length recombinant

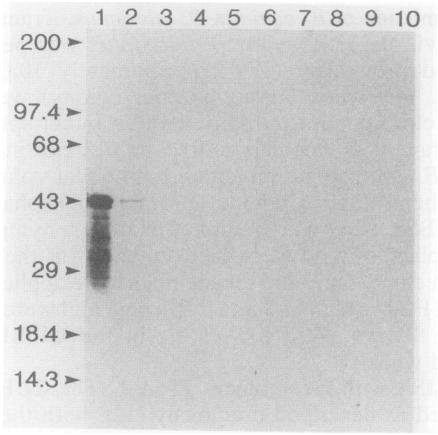

FIG. 1. Immunoblots of lysates from bacteria transformed with either pEma1 or pBluescript and screened with MAb 36/133.97 (lanes 1 to 5) or isotype control MAb (lanes 6 to 10). Lanes 3 and 8, no sample; lanes 1 and 2 and lanes 6 and 7, 0.12 and 0.012 μg of protein from bacterial lysate containing pEma1, respectively; lanes 4 and 5 and lanes 9 and 10 contain 0.12 and 0.012 μg of protein from bacterial lysate containing pBluescript, respectively. Molecular mass standards (in kilodaltons) are given on the left.

EMA-1 is from 32.5 to 40.5 kDa. Potential contributors of molecular mass discrepancies between native and recombinant proteins include secondary processing events of native proteins such as N-linked glycosylation and signal peptide cleavage and anomalous migration of the fusion protein on SDS-PAGE.

CI ELISA with recombinant EMA-1. Eighteen equine serum samples from different countries that were previously shown to be positive for antibody to *B. equi* by CFT and CI ELISA by using native merozoite antigen (12) were retested in the CI ELISA with recombinant EMA-1. All 18 serum samples were positive when recombinant EMA-1 was used (data not shown). An additional 105 equine serum samples that were previously tested for anti-*B. equi* antibodies by CFT were tested by the CI ELISA with recombinant EMA-1. Sample data are given in Table 1. The results from 100 of 105 serum samples agreed in the CFT and CI ELISA (94% concordance). The five discrepant samples were positive by CFT and negative by CI ELISA.

Kinetics of anti-*B. equi* antibody measured by CI ELISA and CFT in experimentally infected horses. Horse H5, which was infected via intravenous inoculation, became positive for antibody to *B. equi* at 2 weeks postinoculation by CFT and at 3 weeks by CI ELISA (Fig. 2). Whereas horse H5 serum remained positive by CI ELISA during weeks 3 through 115, H5 serum became negative by CFT beginning at week 8 and remained negative for the entire test period (Fig. 2). Sera from horses infected via the tick vector *B. microplus* became positive for anti-*B. equi* antibody at 2 weeks postexposure by CFT and at 7 weeks postexposure by CI ELISA (Fig. 3). Although sera from horse H20 remained positive by CFT and CI ELISA through the test period (Fig. 3B), CFT titers in the sera of horse H16 were highly variable and undetectable at weeks 10, 16, 20, and 38 postexposure (Fig. 3A).

DISCUSSION

Results of the present study indicate that a recombinant merozoite antigen (EMA-1) can be used for the detection of antibodies to *B. equi* in the sera of infected horses. Antibodies of diagnostic significance were detected in the sera of horses infected via the tick vector *B. microplus*, by intravenous injection of infected erythrocytes, or by natural exposure. Experimentally infected horses developed and maintained EMA-1-specific antibodies for the entire test period, even though they were not reexposed to *B. equi*.

The assay with recombinant antigen is reliable in that there was complete agreement of test results when sera from horses from 18 countries previously tested by CFT and the

TABLE 1. Sample data from CI ELISA with recombinant EMA-1 and CFT[a]

Serum sample no.	CI ELISA OD[b]	CFT titer[c]	Serum sample no.	CI ELISA OD	CFT titer
280	0.849, 0.754	Negative	296	0.938, 0.971	Negative
282	0.524, 0.614	1:10	297	0.451, 0.367	1:20
283	0.236, 0.272	1:40	298	0.352, 0.363	1:20
285	0.293, 0.327	1:5	299	0.429, 0.504	1:5
286	0.855, 0.892	1:5[d]	300	0.994, 1.087	Negative
287	0.734, 0.827	1:5[d]	301	0.379, 0.390	1:5
288	0.475, 0.473	1:40	302	1.013, 1.072	Negative
289	0.358, 0.347	1:20	303	0.446, 0.442	1:40
290	0.107, 0.092	1:40	304	0.203, 0.235	1:10
291	0.472, 0.511	1:40	305	0.206, 0.195	1:10
292	0.391, 0.396	1:40	306	0.382, 0.396	1:40
293	0.329, 0.339	1:5	307	0.434, 0.430	1:40
294	0.526, 0.565	1:20	308	0.404, 0.424	1:40
295	0.261, 0.327	1:40	309	0.339, 0.395	1:40

[a] CI ELISA and CFT were performed as described in the text.

[b] Sera that reduced the mean of duplicate optical density (OD) values to less than 3 standard deviations below the mean for control horses (<0.7) at a dilution of 1:10 were considered positive. The optical density for control horses at a 1:10 dilution on this test day was 1.00 ± 0.10 (mean ± standard deviation; $n = 6$). The optical density for the isotype control MAb was 0.126, 0.169. Numbers represent duplicate readings.

[c] CFT titers are presented at the highest dilution that yielded a positive result.

[d] Discrepant data were obtained from serum samples 286 and 287. Sera tested positive for antibodies to *B. equi* in CFT but were negative by CI ELISA at dilutions of 1:2 and 1:10.

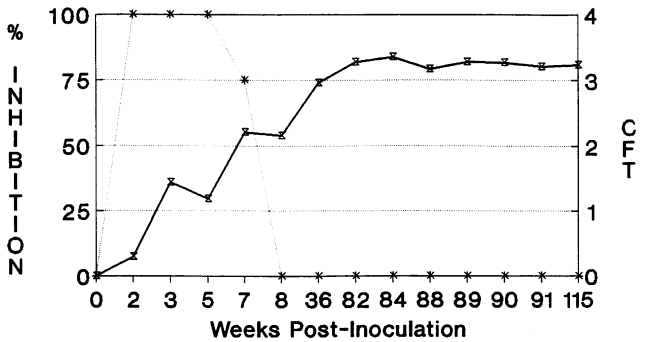

FIG. 2. Development of anti-EMA-1 antibody response in horse H5 as measured by CI ELISA and CFT. Horse H5 was infected with *B. equi* by intravenous inoculation of a stabilate as described in the text. CI ELISA and CFT were performed as described in the text. Percent inhibition (indicated by hourglass symbols) was calculated by the formula {[optical density (preexposure) − optical density (postexposure)]/optical density (preexposure)} × 100. Sera were considered positive at 25% inhibition. CFT values (indicated by asterisks) of 0 to 4 correspond to 100, 75, 50, 25, and 0% lysis, respectively. One hundred percent lysis (0) is a negative result in the CFT. Values of 1 to 4 are considered positive in the CFT.

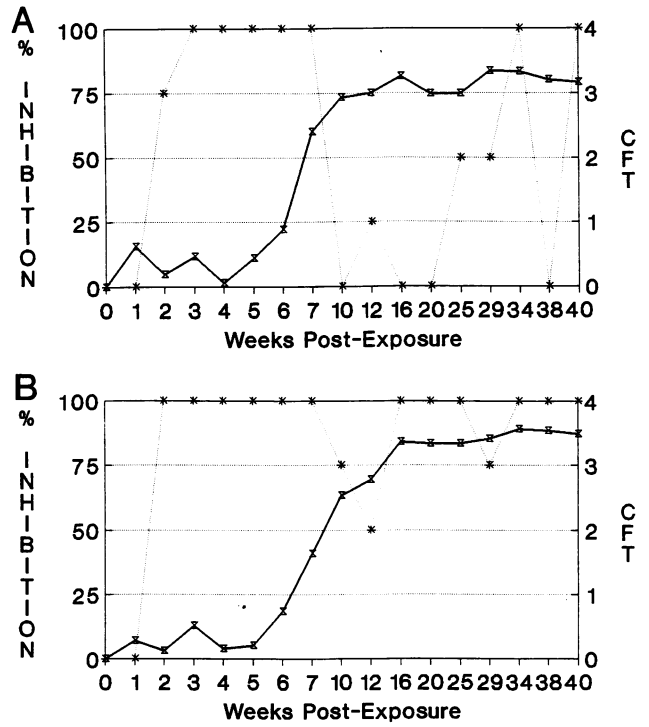

FIG. 3. Development of anti-EMA-1 antibody response in horses H16 (A) and H20 (B) as measured by CI ELISA and CFT. Horses were infected with *B. equi* via the tick vector *B. microplus* as described in the text. Percent inhibition (indicated by hourglass symbols) was calculated by the formula {[optical density (preexposure) − optical density (postexposure)]/optical density (preexposure)} × 100. Sera were considered positive at 25% inhibition. CFT values (indicated by asterisks) of 0 to 4 correspond to 100, 75, 50, 25, and 0% lysis, respectively. One hundred percent lysis (0) is a negative result in the CFT. Values of 1 to 4 are considered positive in the CFT.

native antigen CI ELISA (12) were retested by the recombinant EMA-1 CI ELISA. Also, reasonable concordance (94%) was observed when an additional 105 equine serum samples were tested by CFT and recombinant EMA-1 CI ELISA. This level of concordance is identical to that observed in a previous study that compared the native antigen CI ELISA with CFT (12). The recombinant EMA-1 CI ELISA offers two distinct advantages over the previously described CI ELISA (12): (i) replicate antigen is easily reproduced through expression from the recombinant plasmid, and (ii) the in vitro source precludes the need to infect horses to obtain antigen for the assay.

The finding that horses infected with *B. equi* via *B. microplus* developed CI ELISA-detectable antibody responses 4 weeks later than the horse infected via intravenous inoculation did is most likely due to a difference in the initial infecting dose. However, these data stress the importance of measuring antibody responses in animals infected via tick exposure when the objective is to define parameters for diagnosis. The limited observations of the present study indicate that development of specific antibody to recombinant EMA-1 occurs under minimal conditions of challenge. The specific antibody response to EMA-1, measured by CI ELISA, may develop sooner in horses exposed to more ticks harboring higher numbers of *B. equi*.

Comparison of the development and maintenance of specific anti-*B. equi* antibody by both the CI ELISA and CFT revealed two findings. First, specific antibody was detectable by CFT at 2 weeks postchallenge, regardless of the route of infection. In the horses infected via *B. microplus*, antibody detectable by CFT developed 5 weeks earlier than antibody detectable by CI ELISA. In this and a previous study (12), a limited number of serum samples which were CFT positive and CI ELISA negative were identified. If these sera represent false-negative CI ELISA results, a possible explanation derived from the data obtained in the present study is that the sera were obtained from horses within the first 7 weeks postinfection. Alternatively, the sera may represent false-positive CFT results.

Second, although specific antibody measured by CFT was detectable earlier, CFT results were erratic in two of the horses (Fig. 2 and 3). Horse H5, which was challenged with *B. equi* via intravenous inoculation, became negative by CFT at 8 weeks postinoculation and remained negative by CFT for the 107 weeks that the horse's sera were tested (Fig. 2). Horse H16, which was infected with *B. equi* via tick transmission, was recurrently negative by CFT during the 40 weeks it was tested (Fig. 3). Studies demonstrating variable results by CFT for the detection of equine anti-*B. equi* antibodies were reviewed previously (4). The cause of these variable CFT results is not known; however, fluctuating specific IgG(T) antibody levels may be a contributing factor, because IgG(T) antibodies are known to inhibit complement fixation assays (15).

False-positive results may occur when antigen preparations in other ELISA formats are contaminated with *E. coli*, erythrocyte, or cell culture antigens. The CI ELISA format (1) overcomes antigen purity problems. Since the specificity of the CI ELISA resides solely in the MAb used, the CI ELISA format is well-suited for use with recombinant antigens. Also, the use of recombinant protein precludes the need to infect horses for antigen production. Collectively, the data presented in this report indicate that the CI ELISA with recombinant EMA-1 reliably detects antibody to *B. equi* in the sera of infected horses.

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REFERENCES

1. **Anderson, J.** 1984. Use of monoclonal antibody in a blocking ELISA to detect group specific antibodies to bluetongue virus. J. Immunol. Methods **74**:139–149.
2. **Frerichs, W. M., A. A. Holbrook, and A. J. Johnson.** 1969. Equine piroplasmosis: production of antigens for the complement fixation test. Am. J. Vet. Res. **30**:1337–1341.
3. **Frerichs, W. M., A. A. Holbrook, and A. J. Johnson.** 1969. Equine piroplasmosis: complement fixation titers of horses infected with *Babesia caballi*. Am. J. Vet. Res. **30**:697–702.
4. **Friedhoff, K. T.** 1982. The piroplasms of Equidae—significance for international commerce. Berl. Muench. Tieraerztl. Wochenschr. **95**:368–374.
5. **Golding, A. L.** 1972. Degradation of abnormal proteins in E. coli. Proc. Natl. Acad. Sci. USA **69**:422–426.
6. **Golding, A. L., and A. C. St. John.** 1976. Intracellular protein

degradation in mammalian and bacterial cells: part 2. Annu. Rev. Biochem. **45**:747–803.
7. **Gubler, U., and B. J. Hoffman.** 1983. A simple and very efficient method for generating cDNA libraries. Gene **25**:263–269.
8. **Hanahan, D.** 1985. Techniques for transformation of *E. coli*, p. 109–135. *In* D. M. Glover (ed.), DNA cloning, vol. I. A practical approach. IRL Press, Washington, D.C.
9. **Hirato, K., N. Nonomiya, Y. Uwano, and T. Kuth.** 1945. Studies on the complement fixation reaction for equine piroplasmosis. Jpn. J. Vet. Sci. **7**:197–205.
10. **Holbrook, A. A.** 1969. Biology of equine piroplasmosis. J. Am. Vet. Med. Assoc. **155**:453–454.
11. **Knowles, D. P., L. E. Perryman, W. L. Goff, C. D. Miller, R. D. Harrington, and J. R. Gorham.** 1991. A monoclonal antibody defines a geographically conserved surface protein of *Babesia equi* merozoites. Infect. Immun. **59**:2314–2417.
12. **Knowles, D. P., L. E. Perryman, L. S. Kappmeyer, and S. G. Hennager.** 1991. Detection of equine antibody to *Babesia equi* merozoite proteins by a monoclonal antibody-based competitive inhibition ELISA. J. Clin. Microbiol. **29**:2056–2058.
13. **Knowles, R. C.** 1988. Equine babesiosis: epidemiology, control and chemotherapy. Equine Vet. Sci. **8**:61–64.
14. **Laveran, A.** 1901. Contribution a l'etude de Piroplasma equi. C.R. Soc. Biol. **53**:385–388.
15. **McGuire, T. C., G. L. Van Hoosier, Jr., and J. B. Henson.** 1971. The complement-fixation reaction in equine infectious anemia: demonstration of inhibition by IgG(T). J. Immunol. **107**:1738–1744.
16. **Palmer, D. A., G. M. Buening, and C. A. Carson.** 1982. Cryopreservation of *Babesia bovis* for in vitro cultivation. Parasitology **84**:567–572.
17. **Schein, E.** 1988. Equine babesiosis, p. 197–208. *In* M. Ristic (ed.), Babesiosis of domestic animals and man. CRC Press, Inc., Boca Raton, Fla.
18. **Short, J. M., J. M. Fernandez, J. A. Sorge, and W. D. Huse.** 1988. Lambda ZAP: a bacteriophage with in vivo excision properties. Nucleic Acids Res. **16**:7583–7600.
19. **Stiller, D., W. Goff, D. P. Knowles, L. W. Johnson, and S. Landry.** 1989. *Boophilus microplus* (Canestrini): an experimental transstadial vector of *Babesia equi* (Laveran) to burros, abstr. 179, p. 33. Abstr. 70th Annu Meet. Conference of Research Workers in Animal Disease.
20. **Takacs, B.** 1979. Electrophoresis of proteins in polyacrylamide slab gels, p. 81–105. *In* I. Lefkovits and B. Pernis (ed.), Immunological methods. Academic Press, Inc., New York.
21. **Towbin, H., and H. Gordon.** 1984. Immunoblotting and dot immunoblotting—current status and outlook. J. Immunol. Methods **72**:313–340.
22. **Young, R. A., and R. W. Davis.** 1983. Efficient isolation of genes using antibody probes. Proc. Natl. Acad. Sci. USA **80**:1194–1198.