Platelet function and association of bovine viral diarrhea virus with platelets of persistently infected cattle

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Objective—To determine whether viral involvement with platelets obtained from cattle persistently infected (PI) with bovine viral diarrhea virus (BVDV) is associated with altered platelet function or decreased platelet counts.

Sample Population—Platelets obtained from 8 cattle PI with BVDV and 6 age-, sex-, and breed-matched uninfected control cattle.

Procedure—Manual platelet counts were determined, and platelet function was assessed through optical aggregometry by use of the aggregation agonists ADP and platelet-activating factor. Identification of BVDV in serum and preparations of purified platelets was determined by use of virus isolation tests.

Results—No significant difference in platelet counts was detected between cattle PI with BVDV and control cattle. In response to the aggregation agonists, maximum aggregation percentage and slope of the aggregation curve were not significantly different between cattle PI with BVDV and control cattle. We isolated BVDV from serum of all PI cattle and from purified platelets of 6 of 8 PI cattle, but BVDV was not isolated from serum or platelets of control cattle.

Conclusions and Clinical Relevance—Isolation of BVDV from platelets in the peripheral circulation of cattle immunotolerant to BVDV does not result in altered platelet function or decreases in platelet counts. (*Am J Vet Res* 2005;66:1738–1742)

Bovine viral diarrhea virus (BVDV) is an economically important pathogen of cattle, and BVDV has a worldwide distribution. Acute, postnatal infections with BVDV may be subclinical or result in mild clinical disease[1]; however, a hemorrhagic syndrome characterized by thrombocytopenia has been observed during acute infections with BVDV.[2-5] Although genotype 2 isolates have primarily been associated with BVDV-induced hemorrhagic syndrome, BVDV-1 isolates can also induce thrombocytopenia, as documented by experimental infection with the BVDV-1 isolate NY-1.[6] Platelets play an important role in hemostasis, and hemorrhage may develop during quantitative disorders in which circulating numbers of platelets are low or qualitative platelet defects in which platelets have altered functional characteristics.

In addition to thrombocytopenia, decreased platelet function contributes to hemorrhage observed during acute infection with BVDV-2.[7,8] Although the mechanism of this decreased platelet function is currently unknown, decreased platelet function was only observed in conjunction with isolation of BVDV from platelets, suggesting that the altered platelet function may have been attributable to a direct virus-platelet interaction.[8]

A major consequence of BVDV infection of pregnant cattle is the birth of **persistently infected (PI)** offspring. Infection of susceptible pregnant cattle with noncytopathic BVDV prior to the development of immunocompetence, which typically develops at approximately 120 days of gestation, may result in the birth of a PI calf that is immunotolerant to BVDV.[9] In general, PI cattle have high viral loads and are an important source of viral transmission within and among cattle herds.[10] A physical association between BVDV and platelets in 2 PI heifers was documented[11] through the use of immunofluorescent antibody testing of washed platelet suspensions.

Because thrombocytopenia and hemorrhage are evident in cattle acutely infected with BVDV only during the period when BVDV can be isolated from washed and gel-filtered platelet suspensions,[8] PI cattle provide an opportunity to study the effect of virus-platelet interactions on platelet function and platelet counts. The purpose of the study reported here was to evaluate the association among platelet counts, virus isolation from platelets, and platelet function in PI cattle.

Materials and Methods

Sample population—Samples of serum and platelets were obtained from 14 cattle for use in the study. Eight cattle were PI with BVDV, and 6 uninfected cattle served as control animals. To determine whether the PI cattle were PI with BVDV-1 or -2, genotyping was performed by use of a nested reverse transcription–polymerase chain reaction assay[12] on samples obtained from the 8 PI cattle.

All cattle were housed at Michigan State University or the Ohio Agricultural Disease and Research Center in Wooster, Ohio. Two calves (1 control calf and 1 PI calf) were housed in separate pens in an isolation facility, whereas all other PI cattle were housed on pastures and isolated from all other ruminants. Age- and breed-matched control cattle were housed at the university dairy and beef units at Michigan

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State University. The experiment was performed with approval and in accordance with the guidelines of the Michigan State University All University Committee on Animal Use and Care.

Collection of platelets—For platelet aggregation studies and virus isolation of BVDV from platelets, samples of whole blood were collected from each of the cattle into plastic syringes containing 1.0 mL of 3.8% trisodium citrate for each 9.0 mL of blood.[13] Platelet-enriched plasma was obtained by differential centrifugation performed in accordance with methods described elsewhere.[14] After removal of the platelet-enriched plasma, the remaining sample was centrifuged to yield platelet-depleted plasma. Manual platelet counts were performed by use of a diagnostic reagent system for the enumeration of leukocytes and platelets[a] and a hemacytometer on samples of whole blood and platelet-enriched plasma.

Analysis of platelet aggregation—Aggregation experiments were performed by use of a dual-channel aggregometer.[b] Aggregation agonists used for the experiments were ADP[c] (concentrations of 10, 20, and 100μM) and platelet-activating factor[d] (concentrations of 0.1 and 1.0μM). Platelet-enriched plasma was adjusted to a concentration of 300,000 platelets/μL by the addition of homologous platelet-depleted plasma. For each experiment, the aggregometer was calibrated by use of nonaggregated platelet-enriched plasma to establish the 0% aggregation limit and platelet-depleted plasma to establish the 100% aggregation limit. All aggregation experiments were conducted at least in duplicate and were completed within 5 hours after collection of blood samples. Maximum aggregation percentage and slope of the aggregation curve were measured for each aggregation assay.

Virus isolation—Sera and platelets were obtained from each of the cattle for use in virus isolation procedures. Plasma constituents were removed from platelets by use of a gel-filtration process. Platelet-enriched plasma was obtained as described previously, and prostaglandin E_1[e] was added (1 μL of 1μM prostaglandin E_1/mL of platelet-enriched plasma). Samples were then centrifuged (800 × *g* for 15 minutes). Plasma supernatant was extracted by use of a sterile pipette, and the platelet pellet was resuspended in 1 mL of Hank's balanced salt solution (136mM NaCl, 5.4mM KCl, 0.44mM KH_2PO_4, 0.34mM Na_2HPO_4, and 5.5mM dextrose [pH, 7.4]).[14] The platelet suspension was transferred to a 10-mL polystyrene column containing agarose beads.[f] The eluate was collected, and a manual platelet count was performed. Gel-filtered platelets were adjusted to a concentration of 200,000 platelets/μL by the addition of Eagle minimum essential medium[g] containing 10% fetal equine serum,[h] L-glutamine,[i] penicillin G[j] (100 U/mL), and streptomycin[k] (100 μg/mL). Platelet preparations and sera were stored at –80°C until analyzed.

At the conclusion of the experiment, platelet preparations and sera were thawed, and 25 μL of each sample was inoculated into duplicate wells of 96-well microtiter plates. Each well contained monolayers of bovine turbinate cells in Eagle minimum essential medium containing 10% fetal equine serum, L-glutamine, and antimicrobial. After incubation for 3 days at 37°C in humidified air containing 5% carbon dioxide, bovine turbinate cells were stained for BVDV antigen by use of an immunoperoxidase monolayer assay.[15]

Statistical analyses—Mean values for maximum aggregation percentage and slope of the aggregation curve for each animal were determined and used as the representative value for that animal. Mean ± SEM maximum aggregation percentage and slope of the aggregation curve were determined for the 8 PI and 6 control cattle. Student *t* tests were used to evaluate differences between the PI and control cattle with respect to whole blood platelet count, maximum aggregation percentage, and slope of the aggregation curve. Values of $P < 0.05$ were considered significant.

Results

Virus isolation—We isolated BVDV from serum samples obtained from each of the PI cattle and from preparations of purified platelets obtained from 6 of 8 PI cattle (**Table 1**). We did not isolate BVDV from serum samples or preparations of purified platelets obtained from the control cattle.

Platelet counts and aggregation responses—Platelet counts did not differ significantly ($P = 0.18$) between the PI and control cattle (Table 1). Mean ± SEM for the PI cattle was 564,188 ± 39,413 platelets/μL, whereas mean for the control cattle was 459,417 ± 65,910 platelets/μL.

Platelets from PI and control cattle had typical changes in shape and aggregation response to ADP and platelet-activating factor (**Figure 1**). We did not detect a significant difference in the maximum aggregation

Table 1—Signalment, platelet count, and results of virus isolation (VI) tests for samples obtained from 8 cattle persistently infected (PI) with bovine viral diarrhea virus (BVDV) and 6 age-, breed-, and sex-matched uninfected control cattle.

						VI		
Group	Animal	Age	Sex	Breed	Platelet count (platelets/μL)	Platelets	Serum	BVDV genotype
1	Control	7 y	Female	Angus crossbred	427,000	No	No	NA
2	Control	1 y	Female	Holstein	346,000	No	No	NA
3	Control	1 y	Female	Holstein	336,000	No	No	NA
4	Control	1 y	Castrated male	Holstein	737,000	No	No	NA
5	Control	15 mo	Female	Angus crossbred	565,000	No	No	NA
6	Control	3 d	Male	Holstein	345,500	No	No	NA
1	PI	1 y	Female	Holstein	586,000	Yes	Yes	Type 1
2	PI	1 y	Female	Holstein	405,000	Yes	Yes	Type 1
3	PI	7 y	Female	Angus-Brahma	463,500	Yes	Yes	Type 1
4	PI	7 mo	Female	Holstein	679,000	No	Yes	Type 1
5	PI	1 y	Female	Angus crossbred	707,000	Yes	Yes	Type 1
6	PI	1 y	Female	Angus crossbred	451,000	No	Yes	Type 2
7	PI	1 y	Castrated male	Angus crossbred	622,500	Yes	Yes	Type 1
8	PI	3 d	Male	Holstein	599,500	Yes	Yes	Type 1

NA = Not applicable.

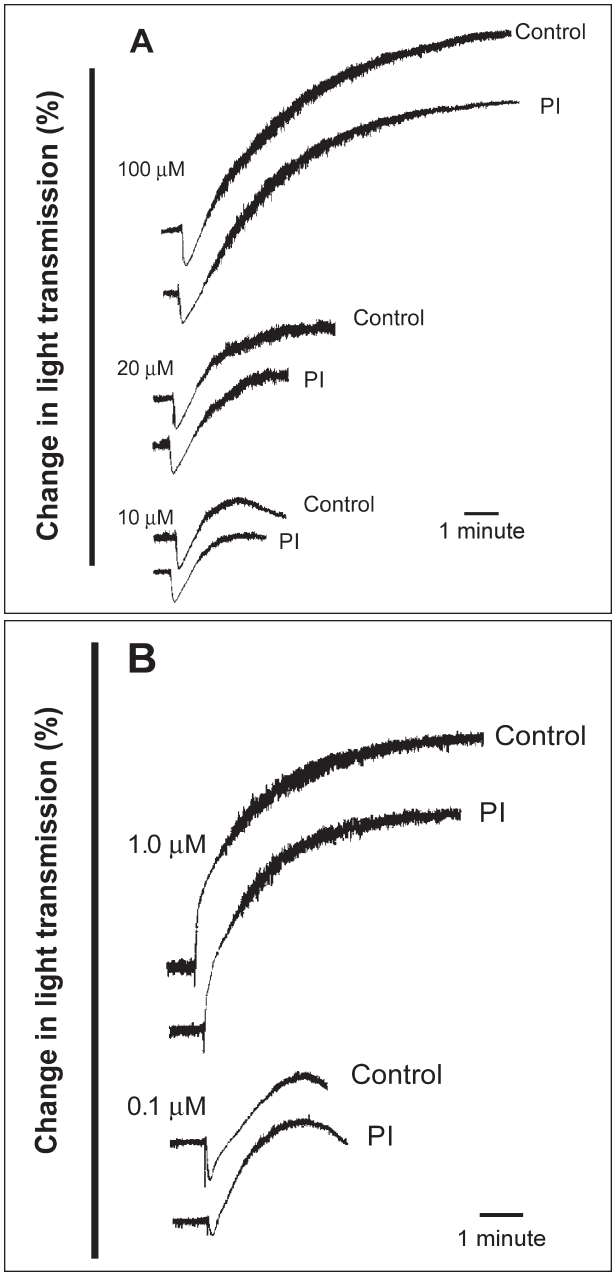

Figure 1—Pairs of aggregometer tracings for suspensions of platelet-rich plasma obtained from a representative heifer persistently infected (PI) with bovine viral diarrhea virus (BVDV) or a representative uninfected control heifer after suspensions were stimulated with ADP at concentrations of 10, 20, and 100μM (A) or platelet-activating factor (PAF) at concentrations of 0.1 and 1.0μM (B).

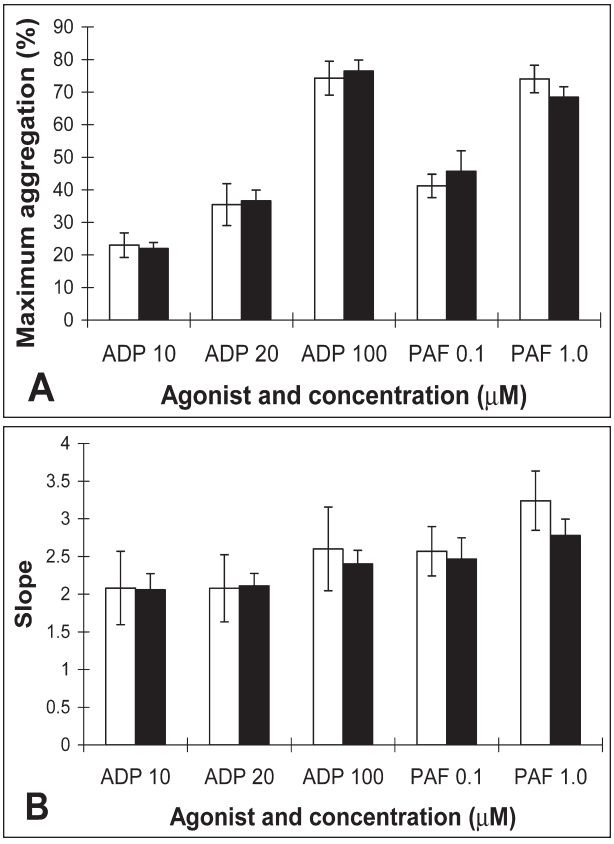

Figure 2—Mean ± SEM maximum aggregation percentage (A) and slope of the aggregation curve (B) induced by addition of various concentrations of ADP or PAF to preparations of purified platelets obtained from 8 cattle PI with BVDV (black bars) and 6 age-, sex-, and breed-matched uninfected control cattle (white bars). Values did not differ significantly ($P < 0.05$) between groups for any agonist at any concentration. *See* Figure 1 for remainder of key.

percentage or slope of the aggregation curve between PI and control cattle (**Figure 2**).

Maximum aggregation percentage was compared between the 6 PI cattle in which BVDV was isolated from purified platelets (ie, platelet-positive group) and the 8 cattle (2 PI cattle and 6 control cattle) in which virus could not be isolated from platelets (ie, platelet-negative group). No differences were observed in the maximum aggregation percentage between platelet-positive and platelet-negative groups of cattle. Mean maximum aggregation percentage did not differ significantly between platelet-positive and platelet-negative groups when ADP was added at concentrations of 10μM (platelet-positive group, 20.9%; platelet-negative group, 22.3%; $P = 0.39$), 20μM (platelet-positive group, 32.3%; platelet-negative group, 38.5%; $P = 0.22$), or 100μM (platelet-positive group, 74.7%; platelet-negative group, 81.7%; $P = 0.16$). Similarly, mean maximum aggregation percentage did not differ significantly between platelet-positive and platelet-negative groups when platelet-activating factor was added at concentrations of 0.1μM (platelet-positive group, 52.4%; platelet-negative group, 41.7%; $P = 0.14$) or 1.0μM (platelet-positive group, 72.8%; platelet-negative group, 73.1%; $P = 0.48$).

Discussion

A biophysical association of BVDV and platelets was documented in the study reported here on the basis of isolation of BVDV from preparations of purified platelets. This association of platelets and BVDV is supported by other studies that have also revealed a relationship in acutely infected animals by use of virus isolation[3,13] and immunofluorescent antibody testing[16,17] and in cattle PI with BVDV by use of immunofluorescent antibody testing.[11] The exact nature of this BVDV-platelet interaction has not been characterized in cattle

persistently or acutely infected with BVDV. Bovine platelets are unique in that they lack an open canalicular system; thus, passive entry and exit of virus through a platelet is unlikely.[18] It is also unlikely that BVDV is involved with platelets through nonspecific adherence. Mixing experiments that used platelets from an uninfected clinically normal calf and BVDV-positive serum have been performed[17]; virus was not isolated after incubation and washing of platelets.

Virus-platelet interactions may be the result of uptake of virus by immature platelets or megakaryocyte infection in the bone marrow. Megakaryocyte infection in the bone marrow by BVDV has been reported[5,19,20] and may be the source of the virus-platelet interaction. In our study, bone marrow biopsy specimens were not evaluated. Isolation of BVDV from purified platelets in 6 of 8 PI cattle may have been the result of megakaryocytes that were infected only in those 6 PI animals. Additional studies in which investigators concurrently evaluate bone marrow samples and platelet suspensions for BVDV may help elucidate the origin of BVDV in platelets.

Thrombocytopenia and altered platelet function in acutely infected cattle without documented platelet dysfunction and in cattle PI with BVDV suggest that the nature of the virus-platelet interaction differs between persistently and acutely infected cattle or that the virus-platelet interaction is not responsible for altered function in acutely infected animals. However, in other studies[5,7] conducted by our laboratory group, we documented that in cattle with experimentally induced acute BVDV-2 infection, the induction of thrombocytopenia and a defect in platelet function were temporally associated with isolation of virus from platelets. If the virus-platelet interaction is responsible for thrombocytopenia or altered platelet function (or both), then cattle PI with BVDV provide a unique opportunity to conduct additional studies on this interaction and its effects. The fundamental defect in cattle PI with BVDV is immunotolerance to BVDV as a result of fetal infection during the period of immune system development.[9]

The observation that platelet counts and platelet function were not affected in cattle PI with BVDV suggests that thrombocytopenia and platelet dysfunction observed in acutely infected cattle may be immunologically based events. Coating platelets with antibodies or deposition of viral antigen-antibody complexes on platelet membranes can trigger immune-mediated destruction of platelets. However, immune-mediated destruction of platelets in the peripheral circulation as the cause of BVDV-induced thrombocytopenia in acutely infected cattle has been considered unlikely on the basis of results of a study[17] in which investigators documented that there was no evidence of platelet-associated antibody or complement in calves infected with the BVDV isolates CD87 and CD89. An immune response directed against bone marrow megakaryocytes or bone marrow support cells may provide an explanation for the thrombocytopenia in acutely infected cattle with a lack of appreciable platelet disturbances in PI cattle. Studies[21,22] have revealed degeneration and necrosis of bone marrow megakaryocytes following BVDV-2 infection of immunocompetent calves. Additional studies are needed to determine whether this degeneration is an immunologically based phenomenon.

Cattle PI with BVDV had typical platelet counts and platelet aggregation responses. The exact nature and role of the BVDV-platelet interaction in the pathogenesis of BVDV-induced platelet disorders in acutely infected cattle require further evaluation. Cattle PI with BVDV are at low risk for bleeding disorders as a result of defects in primary hemostasis.

a. Unopette system, Becton-Dickinson, Franklin Lakes, NJ.
b. Lumi-aggregometer, Chronolog Corp, Havertown, Pa.
c. Adenosine diphosphate, Sigma Chemical Co, St Louis, Mo.
d. Platelet-activating factor-16, Calbiochem Biochemicals, San Diego, Calif.
e. Prostaglandin E_1, Sigma Chemical Co, St Louis, Mo.
f. Sepharose 4B, Sigma Chemical Co, St Louis, Mo.
g. Minimum essential medium, JRH Biosciences, Lenexa, Kan.
h. Fetal equine serum, Sigma Chemical Co, St Louis, Mo.
i. L-glutamine-200mM, Gibco BRL Life Technologies, Grand Island, NY.
j. Penicillin G potassium salt, Sigma Chemical Co, St Louis, Mo.
k Streptomycin sulfate salt, Sigma Chemical Co, St Louis, Mo.

References

1. Baker JC. The clinical manifestations of bovine viral diarrhea virus infection. *Vet Clin North Am Food Anim Pract* 1995;11:425–445.
2. Rebhun WC, French TW, Perdrizet JA, et al. Thrombocytopenia associated with acute bovine virus diarrhea infection in cattle. *J Vet Intern Med* 1989;3:42–46.
3. Bolin SR, Ridpath JF. Differences in virulence between two noncytopathic bovine viral diarrhea viruses in calves. *Am J Vet Res* 1992;53:2157–2163.
4. Corapi WV, Elliott RD, French TW, et al. Thrombocytopenia and hemorrhages in veal calves infected with bovine viral diarrhea virus. *J Am Vet Med Assoc* 1990;196:590–596.
5. Walz PH, Bell TG, Steficek BA, et al. Experimental model of type II bovine viral diarrhea virus-induced thrombocytopenia in neonatal calves. *J Vet Diagn Invest* 1999;11:505–514.
6. Marshall DJ, Moxley RA, Kelling CL. Severe disease following experimental exposure of calves to noncytopathic bovine viral diarrhoea virus New York-1. *Aust Vet J* 1998;76:428–430.
7. Walz PH, Steficek BA, Baker JC, et al. Effect of experimentally induced type II bovine viral diarrhea virus infection on platelet function in calves. *Am J Vet Res* 1999;60:1396–1401.
8. Walz PH, Bell TG, Grooms DL, et al. Platelet aggregation responses and virus isolation from platelets in calves experimentally infected with type I or type II bovine viral diarrhea virus. *Can J Vet Res* 2001;65:241–247.
9. McClurkin AW, Littledike ET, Cutlip RC, et al. Production of cattle immunotolerant to bovine viral diarrhea virus. *Can J Comp Med* 1984;48:156–161.
10. Houe H. Epidemiological features and economical importance of bovine virus diarrhoea virus (BVDV) infections. *Vet Microbiol* 1999;64:89–107.
11. Bezek DM, Stoffregen D, Posso M. Effect of cytopathic bovine viral diarrhea virus (BVDV) superinfection on viral antigen association with platelets, viremia, and specific antibody levels in two heifers persistently infected with noncytopathic BVDV. *J Vet Diagn Invest* 1995;7:395–397.
12. Ridpath JF, Bolin SR, Dubovi EJ. Segregation of bovine viral diarrhea virus into genotypes. *Virology* 1994;205:66–74.
13. Walz PH, Bell TG, Wells JL, et al. Relationship between degree of viremia and disease manifestation in calves with experimentally induced bovine viral diarrhea virus infection. *Am J Vet Res* 2001;62:1095–1103.
14. Steficek BA, Thomas JS, Baker JC, et al. Hemorrhagic diathesis associated with a hereditary platelet disorder in Simmental cattle. *J Vet Diagn Invest* 1993;5:202–207.

15. Meyling A. Detection of BVD virus in viremic cattle by an indirect immunoperoxidase technique. In: McNulty M, MacFerran J, eds. *Recent advances in virus diagnosis*. Boston: Martinus Nijhoff Publishing, 1988;37–46.

16. Bezek DM, Grohn YT, Dubovi EJ. Effect of acute infection with noncytopathic or cytopathic bovine viral diarrhea virus isolates on bovine platelets. *Am J Vet Res* 1994;55:1115–1119.

17. Corapi WV, French TW, Dubovi EJ. Severe thrombocytopenia in young calves experimentally infected with noncytopathic bovine viral diarrhea virus. *J Virol* 1989;63:3934–3943.

18. Zucker-Franklin D, Benson KA, Myers KM. Absence of a surface-connected canalicular system in bovine platelets. *Blood* 1985;65:241–244.

19. Marshall DJ, Moxley RA, Kelling CL. Distribution of virus and viral antigen in specific pathogen-free calves following inoculation with noncytopathic bovine viral diarrhea virus. *Vet Pathol* 1996;33:311–318.

20. Spagnuolo-Weaver M, Allan GM, Kennedy S, et al. Distribution of cytopathic and noncytopathic bovine viral diarrhea virus antigens in tissues of calves following acute experimental infection. *J Vet Diagn Invest* 1997;9:287–297.

21. Scruggs DW, Fleming SA, Maslin WR, et al. Osteopetrosis, anemia, thrombocytopenia, and marrow necrosis in beef calves naturally infected with bovine virus diarrhea virus. *J Vet Diagn Invest* 1995;7:555–559.

22. Ellis JA, West KH, Cortese VS, et al. Lesions and distribution of viral antigen following an experimental infection of young seronegative calves with virulent bovine virus diarrhea virus-type II. *Can J Vet Res* 1998;62:161–169.