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Serum IgG Immunoglobulin Levels are Associated with Reduced PCR Detection of *Mycoplasma bovis* in Naturally Infected American Bison (*Bison bison*)

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ABSTRACT: *Mycoplasma bovis (M. bovis)* is an important pathogen of American bison (*Bison bison*), associated with high morbidity and mortality epizootics of respiratory and reproductive disease. Despite the significant negative impact on bison health, little is known about the kinetics of disease and the host immune response to infection. To address these questions, a cohort of bison calves was created and serially sampled 5 times, once every 2–3 mo, over a 12-mo period. At each sampling period nasal swab samples were collected and tested by PCR for the presence of *M. bovis*. Serum samples were also collected and assessed for *M. bovis*–specific antibodies using both a commercial and an in-house ELISA. Overall, 19/41 bison (46.3%) had positive PCR tests, and 31/41 (75.6%) were seropositive. Over the course of the study, the frequency of PCR-positive nasal swabs and the ELISA scores decreased, although serum samples remained positive for at least 6 mo following the final positive PCR test. Bison were grouped according to results from the in-house ELISA into high-responder (n=7), low-responder (n=5), and seronegative (n=7) groups. *M. bovis*–specific IgG antibody levels were significantly elevated in the high-responder group compared to the low-responder and seronegative groups. The differences were statistically significant for 3/5 sampling periods. A trend toward increased IgG2 levels was observed in the high-responder group. High total IgG responses correlated with a decline in positive PCR tests from nasal swabs. These data provide evidence that a strong humoral response is beneficial and is probably involved in the clearance of *M. bovis* from bison.

Key words: Antibodies, bison, ELISA, IgG, mycoplasma.

INTRODUCTION

Mycoplasma bovis (M. bovis) is a geographically widespread bacterial pathogen of cattle. First isolated in 1961 from a dairy herd in the US experiencing mastitis, *M. bovis* has a significant impact on the health of dairy and beef cattle in most countries worldwide (Hale et al. 1962; Dudek and Szacawa 2020). The bacterium is associated with the multifactorial bovine respiratory disease complex (BRDC) causing respiratory disease, and with chronic pneumonia and polyarthritis syndrome (Maunsell et al. 2011). Additionally, *M. bovis* can cause mastitis, otitis media, keratoconjunctivitis, and reproductive disorders. Control of the disease is challenging because of the multifactorial etiology, varying clinical signs, and a chronic phase that includes the shedding of bacteria from asymptomatic carriers (Wilson et al. 2007; Nicholas et al. 2008; Calcutt et al. 2018).

In contrast to its involvement with BRDC in cattle, *M. bovis* is a primary respiratory pathogen of American bison (*Bison bison*; Register et al. 2018). First described in the early 2000s, *M. bovis* infection of bison has repeatedly caused large outbreaks characterized by high morbidity and mortality, reaching as high as approximately 25%, in both the US and Canada (US Department of Agriculture [USDA] 2013), with up to 45% mortality in adult cows (Janardhan et al. 2010). *M. bovis* infection causes a disease characterized by pneumonia, arthritis, pleuritis, and abortion. Clinical signs observed in infected bison include lethargy, lameness, joint swelling, coughing, and loss of body condition

(Bras et al. 2016). Control efforts have been hindered by the lack of a commercially available vaccine licensed for use in bison, lack of effective antimicrobials, incomplete knowledge about how the disease is introduced into bison herds, and lack of a commercially available diagnostic assay validated for use with bison samples. The lack of antemortem diagnostic tests impairs the ability to translocate live animals safely to maintain gene flow, threatening conservation goals of bison managers in addition to the direct conservation impacts of *M. bovis* in affected herds.

Despite the significant impact on bison health and conservation, little information is available regarding bacterial shedding and antibody kinetics in response to *M. bovis* infection. The objectives of this study were to characterize the infection status and serum antibody responses in a cohort of bison naturally exposed to *M. bovis*. Results presented herein describe the longevity and the serum antibody response over a 12-mo period.

MATERIALS AND METHODS

Study site and population

We sourced a cohort of animals from a closed herd of American bison residing on privately owned pasture located in north-central South Dakota, US. An outbreak of *M. bovis* infection, first noted with lameness and deaths, occurred in the herd from September 2021 through January 2022. At the time of outbreak onset, the source herd totaled approximately 850 animals. The mortality rate for this outbreak was determined to be 45% of the adult cows, in addition to a smaller number of yearlings and adult bulls. A convenience sample of bison ranging in age from calves (<12 mo of age) to 18 yr of age were captured from the source herd in February 2022. To assess infection dynamics in juveniles, calves and yearlings were retained following the initial sampling period, and adults were released. A total of 41 bison were captured and housed in a separate corral sequestered from the main herd. The cohort was comprised predominantly of female bison (n=27), with approximately one-third males (n=14). The largest age group was weaned calves (n=20), less than 1 yr of age, followed by yearlings (n=12), between 1 and 2 yr old. Six adult female bison initially captured ranged from 8 to 18 yr old. The largest demographic represented in the cohort was female calves (n=14). Complete demographic data could not be collected for one adult female (age unknown). Clinical signs were not systematically assessed in the study animals, as bison mask clinical signs to avoid predation, particularly in the presence of humans.

Between March 2022 and February 2023, the cohort was sampled approximately every 2–3 mo (five samplings). For sampling, bison were restrained in a squeeze chute and head gate with neck extender, which is standard practice for bison husbandry. Shallow nasal swabs were collected with standard polyester tipped applicators (Puritan Medical Products Company, Guilford, Maine, USA), after which deep nasopharyngeal swabs were collected using double guarded uterine swabs (Kalayjian Industries, Signal Hill, California, USA). Swabs were placed in tubes containing 1 mL tryptic soy broth, 15% glycerol, and held at −20 C until processing. Blood was collected from the jugular vein using a 2.54-cm, 18-gauge needle directly into serum separator tubes (Vacutainer SST, Becton Dickinson, Franklin Lakes, New Jersey, USA). Serum was separated from blood by centrifugation at 1,200 × G for 15 min, aliquoted, then stored at –20 C for analysis.

Mycoplasma bovis real-time quantitative PCR (qPCR)

We extracted DNA from nasal swabs using the DNeasy Blood & Tissue kit (Qiagen, Germantown, Maryland, USA) via hand extraction, Qiacube (Qiagen), or the KingFisher Flex Purification system (ThermoFisher, Waltham, Massachusetts, USA). Real-time quantitative PCR (qPCR) targeting the *uvrC* gene of *M. bovis* was performed on extracted DNA in triplicate using primers, probe, and cycling conditions as described (Rossetti et al. 2010; Johnson et al. 2022). Briefly, PCR reactions consisted of 5 µL of sample (20–100 ng of template DNA), 1 µL of 20 µM forward and reverse primers, 1 µL of 8 µM *uvrC* probe, 12.5 µL Path-ID qPCR Master Mix (ThermoFisher), 0.5 µL Xeno Internal Control Positive LIZ Assay (ThermoFisher), and nuclease-free water to 25 µL. Cycling conditions were 95 C for 10 min, 40 cycles of 95 C for 15 s, and 56 C for 60 s. Samples were considered positive if the Ct values ≤36.

Serological testing

Initial testing of serum was conducted using a commercial ELISA, the Monoscreen AbELISA

Mycoplasma bovis indirect, monowell ELISA kit (Bio-X Diagnostics S.A., Rochefort, Belgium), following the manufacturer's instructions regarding procedure and interpretation of results. Serum samples from animals with at least one positive nasal swab PCR test across two different sampling periods were then assayed for total IgG, IgM, and IgG2, and the results were compared to those collected from seronegative, PCR-negative bison. For this we used an in-house ELISA that incorporates whole-cell *Mycoplasma* protein extracts as the antigen as described (Register et al. 2013) with minor modifications. Briefly, three bison isolates (NADC1, NADC15, and NADC16) were grown for 18–24 h at 37 C, 5% CO_2 in pleuropneumonia-like organism (PPLO) medium supplemented with 10 g/L of yeast extract and 20% horse serum. Bacterial cells were washed with phosphate-buffered saline (PBS) and resuspended in a 2% Tween-20, PBS solution to generate a cell extract. A total of 1 µg of protein extract was diluted in 0.1 M carbonate-bicarbonate buffer, pH 9.6 (Millipore Sigma, Burlington, Massachusetts, USA) and used to coat Immulon 2HB 96-well plates overnight at 4 C. Plates were washed with Tris-buffered saline, Tween-20 (10 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.5) and blocked using a commercial blocking reagent, StartingBlock blocking buffer (ThermoFisher). Bison serum samples were diluted to 1:100 in 0.05% Tween-20, PBS and allowed to react with antigens for 1 h at room temperature. Following three washes, 100 µL of horseradish peroxidase (HRP) conjugated secondary reagent diluted in blocking reagent was added to wells and incubated for 1 h at room temperature. After a final three washes, plates were incubated with SureBlue TMB 1-component peroxidase substrate (SeraCare Life Sciences, Milford, Massachusetts, USA) for 10 min prior to the addition of and equal volume of TMB Stop Solution (SeraCare Life Sciences). Absorbance was measured at 405 nm on a FlexStation 3 multimode microplate reader (Molecular Devices, San Jose, California, USA). Different HRP conjugated secondary reagents were utilized to assess specific antibody isotypes and isotype subclasses. Protein-G was used to detect total IgG specific to *M. bovis* antigens, and isotype-specific antibodies were used to detect IgG2, and IgM; sheep anti-bovine IgG2, and sheep anti-bovine IgM heavy chain (Fortis Life Science, Boston, Massachusetts, USA).

Statistical analysis

We used GraphPad Prism 9 to generate bar graphs for data visualization (GraphPad Software, San Diego, California, USA). We used SAS (version 9.4, SAS Institute Inc., Cary, North Carolina, USA) for the general linear models (GLM) analysis of variance (ANOVA) to evaluate the fixed effect of group status, sampling date, and their interaction on results from the in-house, isotype, and commercial ELISAs, and the nasal swab PCR. The models included the effects of date (March 2022, June 2022, August 2022, November 2022, and February 2023), status (high, low, or negative PCR result), and their interaction. Mean comparisons of significant effects were done using the predicted differences option. Significance was set at $P<0.05$.

RESULTS

Using the commercial ELISA, positive serum samples were identified at each sampling period over the 12-mo sampling period (Fig. 1A). The frequency of ELISA scores >3, indicating high levels of *M. bovis*–specific antibody, was highest at the first sampling in March 2022, with gradual reductions at each subsequent sampling period and reaching a minimum at the final sampling in February 2023 (Fig. 1B). The highest frequency of PCR-positive nasal swabs was observed during the first sampling period in March 2022. The frequency of PCR-positive nasal swabs declined in the following two sampling periods, ending at approximately 20% in November 2022 and February 2023 (Fig. 1C).

Assessment of total IgG using the in-house ELISA revealed two groups of respondents from the subset of bison with at least one positive nasal swab PCR test across two different sampling periods: A high-responder group (optical density [OD] 450 nm >1.1); and a low-responder group (OD 450 nm ≤1.1). Group composition and test results for individual animals are listed in Table 1. Total IgG levels were significantly higher in the high-responder group compared to the low-responder and seronegative groups for the March, June, and August 2022 sampling periods and significantly different

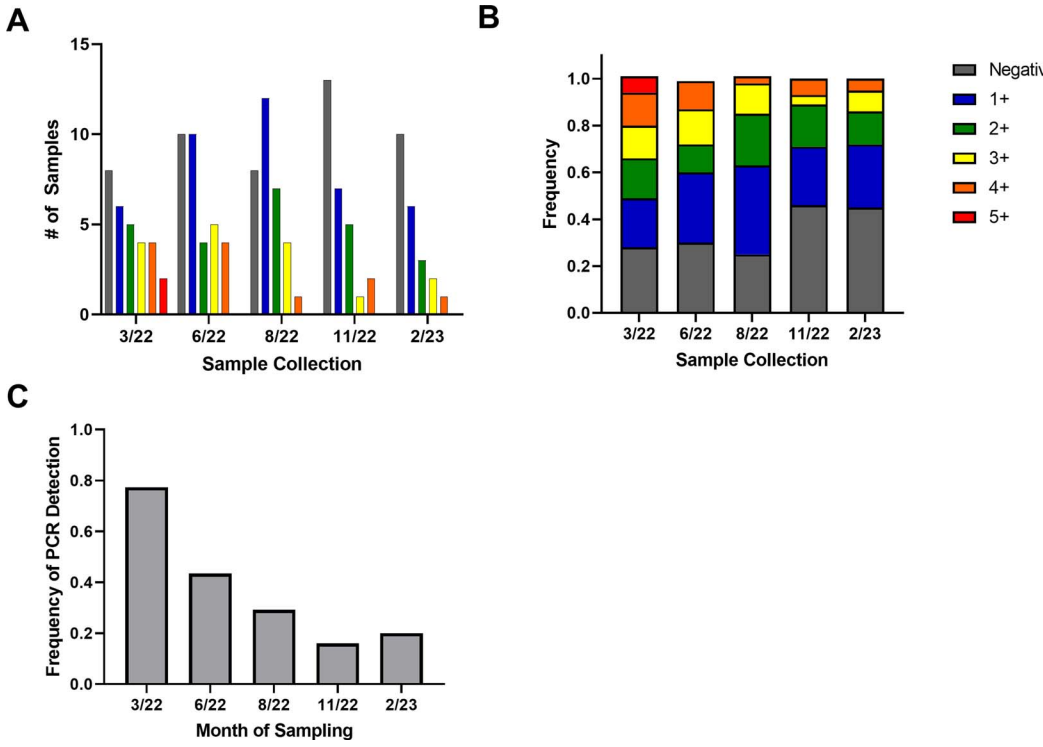

FIGURE 1. Commercial antibody ELISA and nasal swab qPCR results from a cohort of American bison (*Bison bison*) naturally infected with *Mycoplasma bovis*. (A) Total number of samples for each ELISA scores from the BioX K302 *M. bovis* test by sampling. The ELISA scores range from 1 to 5, with 5 indicating the highest levels of specific antibodies. (B) Frequency of each ELISA score from each sampling. (C) Deep and shallow nasal swab *uvrC* qPCR results. C_t values below *x* were considered positive.

from the seronegative group at the November sampling (Fig. 2A). No statistically significant difference was observed at the February 2023 sampling between the high- and low-responder group. The only statistical difference between the low-responder and seronegative groups was observed for the March sampling. In the high-responder group, IgG levels remained relatively static for at least 5 mo (March–August samplings), followed by a gradual decline observed in samples collected at the November and February samplings. Conversely, in the low-responder group, initial IgG levels were stable for the first two samplings (March and June) followed by slight increases at the subsequent three samplings (August, November, February).

To characterize the antibody kinetics in *M. bovis*–infected bison further, IgG2 titers were assessed using the in-house ELISA (Fig. 2B). In the high-responder group, IgG2 kinetics followed a trend close to that observed for total IgG, though the overall antibody levels were lower. The highest observed IgG2 levels were recorded for samples collected during the initial sampling (March 2022) and remained relatively stable following a slight decline after the June sampling, and ultimately were undetectable at the conclusion of the study. In the low-responder group IgG2 levels were stable across four of the five sampling periods throughout the study. For all three groups at all sampling periods, *M. bovis*–specific IgM titers were undetectable using the in-house ELISA (data not shown). Grouping the results of the commercial ELISA kit by high and low responders also revealed similar trends in antibody kinetics as compared to those obtained for total IgG, though the ELISA scores of the high-responder group decreased immediately following the initial (March) sampling (Fig. 2C). In contrast, the

TABLE 1. Results of antibody ELISA and nasal swab PCR *Mycoplasma bovis* testing for individuals in a cohort of naturally infected American bison (*Bison bison*).

				March 2022		June 2022		August 2022		November 2022		February 2023	
Group[a]	Animal ID	Sex	Age	ELISA[b]	Nasal swab[c]	ELISA	Nasal swab	ELISA	Nasal swab	ELISA	Nasal swab	ELISA	Nasal swab
High responder	Blue 01	F	2	4+	Positive	2+	Positive	1+	Negative	Negative	Negative	1+	Negative
	Blue 02	M	2	2+	Positive	1+	Positive	2+	Positive	1+	Negative	NT	Negative
	Green 29	F	Calf	NT[d]	Positive	3+	Positive	2+	Negative	2+	Negative	2+	Negative
	Green 32	F	1–2	3+	Positive	3+	Positive	3+	Positive	2+	Negative	Negative	Negative
	Red 01	F	Calf	4+	Negative	4+	Positive	4+	Positive	4+	Positive	4+	Negative
	Red 09	M	1	5+	Positive	4+	Positive	3+	Negative	4+	Negative	NT	NT
	Red 10	M	2	NT	Positive	4+	Negative	3+	Negative	2+	Negative	NT	NT
Low responder	Green 24	F	Calf	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Positive
	Green 25	F	Calf	4+	Positive	3+	Negative	3+	Positive	2+	Positive	3+	Positive
	Green 33	F	Calf	2+	Positive	3+	Negative	2+	Positive	3+	Positive	3+	Negative
	Green 38	F	1	3+	Positive	2+	Positive	2+	Negative	1+	Negative	1+	Negative
	Red 11	M	1	1+	Positive	NT	NT	Negative	Positive	NT	Positive	2+	Positive
Seronegative,	Blue 03	M	1–2	NT	NT	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
PCR negative	Green 16	M	Calf	Negative	Negative	Negative	Negative	1+	Negative	Negative	Negative	Negative	Negative
	Green 27	F	Calf	NT	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
	Green 34	F	Calf	Negative	Negative	Negative	Negative	1+	Negative	NT	Negative	Negative	Negative
	Red 06	M	Calf	Negative	Negative	1+	Negative	Negative	Negative	Negative	Negative	NT	NT
	Red 07	M	2	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	NT	NT
	Red 08	M	Calf	NT	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Seropositive,	Blue 04	M	1–2	1+	Negative	1+	Negative	2+	Negative	1+	Negative	NT	NT
PCR negative	Green 07	M	1–2	Negative	Negative	1+	Negative	1+	Negative	1+	Negative	NT	NT
	Green 08	M	1	Negative	Negative	1+	Negative	1+	Negative	Negative	Negative	Negative	Negative
	Green 15	F	Calf	1+	Negative	1+	Negative	1+	Negative	Negative	Negative	Negative	NT
	Green 17	M	Calf	Negative	Negative	2+	Negative	Negative	Negative	Negative	Negative	Negative	NT
	Green 20	F	Calf	1+	Negative	Negative	Negative	1+	Negative	Negative	Negative	Negative	NT
	Green 21	F	Calf	NT	Negative	2+	Negative	1+	Negative	1+	Negative	1+	Negative
	Green 22	F	Calf	NT	Negative	1+	NT	1+	Negative	1+	Negative	1+	NT
	Green 28	F	Calf	1+	Negative	1+	Negative	1+	Negative	1+	Negative	Negative	NT

TABLE 1. Continued.

				March 2022		June 2022		August 2022		November 2022		February 2023	
Group[a]	Animal ID	Sex	Age	ELISA[b]	Nasal swab[c]	ELISA	Nasal swab	ELISA	Nasal swab	ELISA	Nasal swab	ELISA	Nasal swab
Other	Green 6	F	1	2+	Positive	1+	Negative	1+	Negative	Negative	Negative	1+	Negative
	Green 09	F	12	NT	Negative	1+	Negative	1+	Negative				
	Green 10	F	8	5+	Negative								
	Green 11	F	10	4+	NT								
	Green 13	F	18	3+	Negative								
	Green 14	F	18	4+	Positive								
	Green 18	F	1	2+	Positive	4+	Positive						
	Green 19	F	Adult		Positive								
	Green 23	F	Calf	NT	Negative	Negative	Negative						
	Green 26	F	Calf	NT	Negative	Negative	Negative						
	Green 31	F	Calf	2+	Positive	3+	Positive	2+	Positive				
	Red 02	F	1	1+	Negative	NT	Positive	2+	NT	2+	NT		
	Red 03	F	15	3+	Positive								

[a] Groups based on commercial ELISA result.
[b] Commercial ELISA, BioX K302 Mycoplasma bovis Monowell ELISA Kit (BioX, Rochefort, Belgium).
[c] RT-PCR results of deep and shallow nasal swab samples.
[d] NT=not tested.

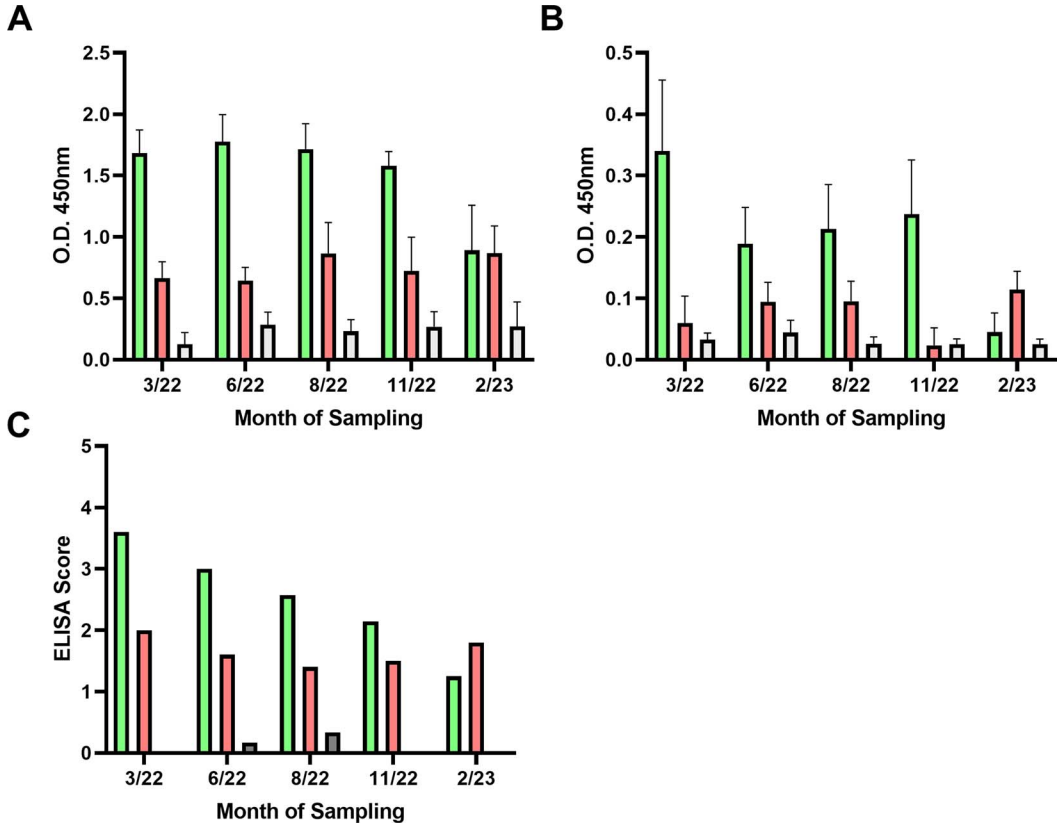

FIGURE 2. Serum IgG levels in high and low responders in a cohort of American bison (*Bison bison*) naturally infected with *Mycoplasma bovis*. Serum samples from bison with at least two positive PCR tests were assayed by in-house ELISA against *M. bovis* Tween-20 extracts. An optical density (OD) at 450 nm >1.1 for the total IgG ELISA was used to populate the high-(green) and low-(red) responder groups. Samples from seronegative and PCR-negative bison were used as negative controls (gray). (A) *M. bovis*–specific total IgG as determined by the in-house ELISA. A significant difference in the OD at 450 nm was observed between the high responders, low responders, and negative controls. The high levels of IgG were maintained for at least 5 mo. (B) IgG2 levels were increased in the high-responder group compared to the low-responder group. (C) ELISA scores from the commercial BioX K302 *M. bovis* test kit parsed by group.

commercial ELISA scores of the low-responder group were relatively stable for the duration of the study.

Comparing qPCR between the high- and low-responder groups, nasal swab samples from bison in the high-responder group tested positive for *M. bovis* with a high frequency (0.86) at the first sampling (Fig. 3); the frequency then gradually declined during the June, August, and November samplings, with all nasal swab samples collected from bison in this group testing negative by the final sampling in February 2023. In the low-responder group, all nasal swabs tested positive for *M. bovis* (1.0) at the March 2022 sampling period, followed by a decline in positive tests. In contrast to the high-responder group, the overall frequency of PCR-positive nasal swabs remained relatively high (0.5) for the duration of the study.

Using a generalized linear model ANOVA, group status was the sole factor ($P<0.0001$), rather than date or an interaction between the two ($P=0.586$ and $P=0.241$, respectively), influencing differences between the total IgG antibody levels, assessed with the in-house ELISA, over the duration of the study(Fig. 4A). Similar

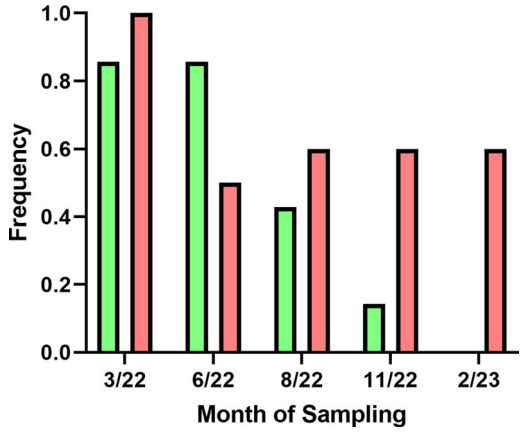

FIGURE 3. Frequency of *Mycoplasma bovis*–positive nasal swabs from high (green) and low (red) serological responders in a cohort of naturally infected American bison (*Bison bison*). Nasal swabs collected from bison were screened for the presence of the *M. bovis* via qPCR targeting the *uvrC* gene. C_t values <37 were considered positive.

findings were obtained for IgG2 (Fig. 4B) and commercial (Fig. 4C) ELISAs where group status was the factor found to contribute significantly ($P<0.001$) to difference in detected antibody levels. For nasal swab PCR, group status had the greatest effect on nasal swab PCR ($P<0.001$), followed by sampling date ($P<0.0052$), then the group status–sampling date interaction ($P<0.0133$; Fig. 4D).

DISCUSSION

Our study provides longitudinal data on bacterial shedding and serum antibody kinetics in a cohort of North American bison calves naturally infected with *M. bovis*. As presented in Table 1, our cohort was comprised predominantly of female calves and yearlings. Previous studies have found adult bison cows (>3 yr of age) to be the primary demographic group affected by *M. bovis*, followed by adult bulls, with yearlings and calves being affected to lesser extents (Bras et al. 2016, 2017a), although these data were based on the observation of clinical signs and not laboratory testing. The adult bison that were initially included in the cohort were released because they were not the population of interest for this study. The high-responder group included a greater number of yearling bison ($n=4$, 57.1%) compared to the low-responder ($n=2$, 40%) and seronegative ($n=2$, 28.5%) groups (Table 1), which contained a higher number of calves. More research is required to understand infection, shedding, and serologic dynamics in younger age classes of bison and additional studies with larger groups will determine if age is a factor for clearance of *M. bovis* following infection.

There have been no previous longitudinal studies on *M. bovis*–specific antibody kinetics in American bison. Currently, the duration of *M. bovis*–specific antibodies in bison is unknown. Previous studies have shown bison to be seropositive in the absence of both clinical disease and positive PCR test, suggesting that *M. bovis*–specific antibodies may be detected for an undefined period following infection or exposure (Bras et al. 2017b; Register et al. 2021). We found that serum antibodies remained detectable by commercial and in-house ELISA for at least 6 mo following detection of *M. bovis* DNA by PCR, although there was a reduction in the number of positive samples with time. In one study, up to 41% of bison from herds with a history of disease due to *M. bovis* were seropositive, and up to 9% of bison were seropositive from herds with no history of disease (Bras et al. 2017b). Of note, several of the seronegative samples tested positive with a score of 1, though for bison sera, that result can indicate a false positive (Register et al. 2013). It is also important to note the potential for cross-reactivity of bison sera with other *Mycoplasma* spp. Register et al. (2021) noted that the culture of *Mycoplasma bovirhinis*, *Mycoplasma dispar*, and *Mycoplasma bovoculi* from seropositive bison, indicating that cross-reactivity of antibody could be produced in response to infection with these nonpathogenic *Mycoplasma* spp. In our study, nine bison were seropositive despite repeated negative PCR tests; this might indicate that these animals had been infected with *M. bovis* previously, before the first sampling, or that they were infected with *M. bovirhinis* or other *Mycoplasma* spp. A major question remains as to whether prior infection will protect bison

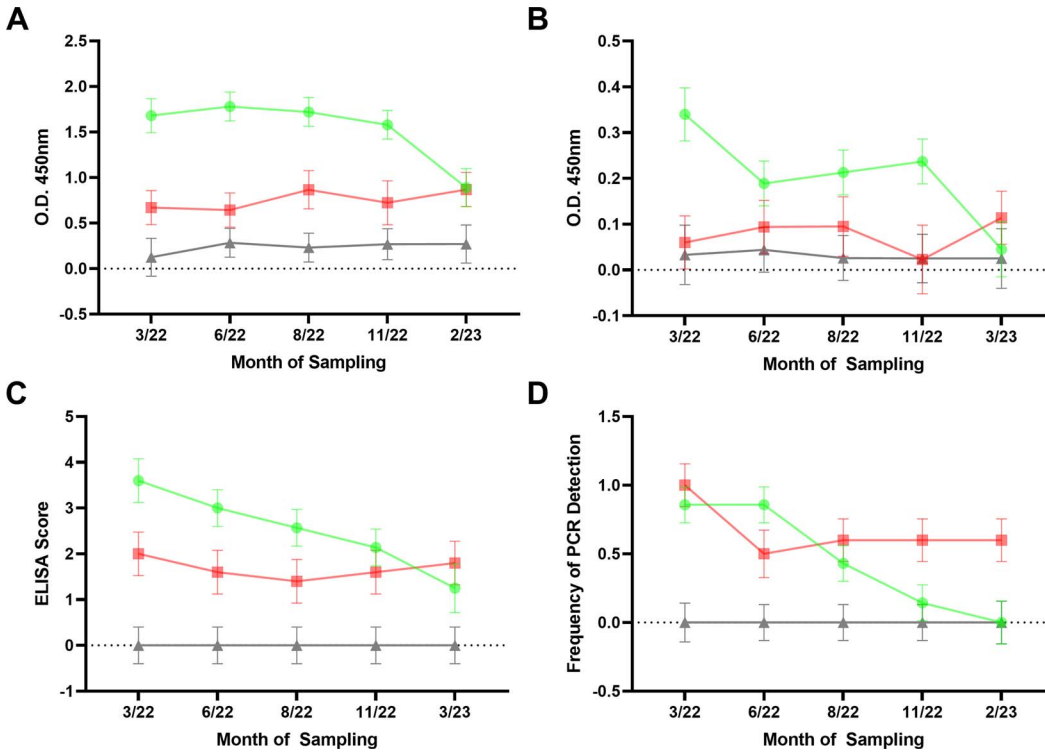

FIGURE 4. Interaction of collection date and group status on antibody response and *Mycoplasma bovis*–positive nasal swab tests in cohort of naturally infected American bison (*Bison bison*). Generalized linear model for an in-house ELISA (A), IgG2 ELISA (B), commercial ELISA (C), and nasal swab PCR (D). High responder (green), low responder (red), and negative (gray) are listed for each. Group status (high or low responder) was the sole factor with a significant effect ($P<0.0001$) on antibody levels for all three ELISA assays. In contrast, both sampling date and group status had significant effects ($P<0.0052$ and $P<0.0001$, respectively) on PCR detection of *M. bovis*.

from a subsequent infection with *M. bovis* or if pre-existing immunity predisposes bison to enhanced disease. Future laboratory and field studies investigating the impact and duration of natural immunity for protection from reinfection are of utmost importance. Further, serological assays for the detection of prior *M. bovis* infection in bison would be a useful diagnostic tool, and understanding bison antibody kinetics and longevity are critical for the development of bison-specific serology assays.

In our study, high serum IgG levels correlated with clearance of *M. bovis* from the upper respiratory tract as determined by nasal swab PCR tests. Additionally, an increase in serum IgG2 antibody was detected in bison in the high-responder group. In cattle, high IgG titers have been shown to correlate with reduced disease and resistance to infection (Howard et al. 1980; Nicholas et al. 2002), suggesting a role for humoral antibody in protection. In experimental challenge studies in cattle, high serum IgG levels were produced following challenge, although IgG1 was the predominant isotype detected and no detectable increase in IgG2 levels were observed (Vanden Bush and Rosenbusch 2003). Vaccination studies have shown that adjuvants, including Emulsigen and CpG 2007 oligonucleotide, induced a more balanced IgG1/IgG2 antibody repertoire when administered with *M. bovis* extracts, membrane fractions, and purified proteins (Mulongo et al. 2013; Prysliak et al. 2017). In these studies, no protection was observed when *M. bovis* extracts or membrane fractions were used as vaccine

antigen, suggesting that other aspects of the immune response are required for protection. In cattle, the immune response to *M. bovis* infection is biased towards a Th17 response which is insufficient in preventing disease (Prysliak et al. 2018; Chao et al. 2019). Recent studies have shown mycoplasma lipoproteins are responsible for the polarization towards a Th17 response and vaccine-enhanced disease (Mara et al. 2020, 2022). The IgG2 isotype is associated with opsonization and killing of bacteria by macrophages and neutrophils and is thought to contribute to a protective immune response (Howard 1984). The heightened IgG2 levels in bison in the high-responder group suggests a protective role for this antibody isotype in bison that is potentially mediated by opsonization of bacteria, though further work is needed to assess if class-switching to IgG2 is enhanced in recovered bison. A previous study has shown that *M. bovis* inhibits proliferation and delays the induction of apoptosis in bison peripheral blood mononuclear cells and macrophages, respectively (Suleman et al. 2016). Additionally, there are conflicting reports regarding the role of antibody-mediated complement killing in protection from *M. bovis* (Zhang et al. 2019; Prysliak et al. 2023). Further research characterizing the immune response, and more specifically, investigating the molecular mechanisms underlying the initiation of protective immune responses and the biological activities of specific antibody isotypes, and the targets of these antibodies, is critical to understanding the magnitude and mechanism of antibody-mediated protection against *M. bovis* in bison. Development of sensitive and specific serologic tests, and associated diagnostic and management tools, are needed to assist the continued restoration of wild bison in North American and associated cultural, economic, and conservation values.

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