

HUMORAL IMMUNE RESPONSE TO *KLEBSIELLA* SPP. IN NEW ZEALAND SEA LIONS (*PHOCARCTOS HOOKERI*) AND THE PASSIVE TRANSFER OF IMMUNITY TO PUPS

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ABSTRACT: During the 2001–02 and 2002–03 breeding seasons, epizootics of *Klebsiella pneumoniae* resulted in a dramatic increase of pup mortality in New Zealand sea lions (*Phocarctos hookeri*; NZSLs) on Enderby Island (Auckland Islands). To estimate the prevalence of infection in the NZSL population, a serologic test was developed using a Western blot and a polysaccharide antigen derived from a *K. pneumoniae* isolate from a NZSL pup. All archived serum samples collected between 1997 and 1998 and 2004 and 2005 at Sandy Bay Beach rookery, Enderby Island, were tested (314 pups and 302 adult females). Anti-*Klebsiella* antibodies were detected throughout this period, but overall, only 16% of NZSL pups between birth and 5 mo of age were seropositive compared with 95.7% of adults. There was no apparent change in antibody prevalence as a result of the two epizootics. A method to determine total immunoglobulin G (IgG) levels in sea lion serum also was developed to investigate passive immunoglobulin transfer to neonates and development of an acquired immune response. The IgG concentration was significantly lower in pups (median 2.1 mg/ml) than in adult females (median 80 mg/ml). Based on serologic results, it was not possible to determine whether *K. pneumoniae* was an endemic or a novel pathogen to the NZSL population because the test was not able to discriminate between *Klebsiella* species. However, this study suggested that the transfer of passive immunity to neonates was very low in the NZSL, especially for anti-*Klebsiella* antibodies.

Key words: Immunoglobulin G, *Klebsiella* spp., New Zealand sea lion, passive immunity, *Phocarctos hookeri*, Western blot.

INTRODUCTION

The New Zealand sea lion (*Phocarctos hookeri*; NZSL) is one of the world's rarest pinnipeds, and it breeds almost exclusively on the Auckland Islands (50°S, 166°E). The 2001–02 and 2002–03 breeding seasons on Enderby Island (Auckland Islands) were marked by high pup mortality (31.3% in 2001–02 and 21.7% in 2002–03) compared with previous years (10.9%; Castinel et al., 2007a), with a dramatic increase in the proportion of bacterial infections diagnosed at necropsy (Wilkinson et al., 2006). From 2001 to 2002, an opportunistic enterobacterium identified as *Klebsiella pneumoniae* was consistently isolated in pure culture from internal

organs of dead pups (Castinel et al., 2007b).

The sudden increase in pup mortality on the Auckland Islands in 2001–02 and 2002–03 suggested that the epizootic strain of *K. pneumoniae* could have been a novel pathogen introduced to a naïve NZSL population. To confirm this hypothesis, serum antibodies to *K. pneumoniae* would have to have been absent or present at very low frequencies in the NZSL population before the 2001–02 and 2002–03 breeding seasons.

Generally, *Klebsiella* species are widely distributed, and they are diagnosed by bacterial cultures. The detection of serum antibodies to *Klebsiella* spp. has been limited to research applications rather than

TABLE 1. Distribution of serum samples from New Zealand sea lions by age, breeding season, and survival, between 1997–98 and 2004–05 on the Auckland Islands. There are no data available for the 2003–04 breeding season.

Status and age	Yr							Total	
	1997–98	1998–99	1999–2000	2000–01	2001–02	2002–03	2004–05		
Live pups	Birth–1 mo	—	—	—	—	—	23	23	
	1–3 mo	—	—	—	—	—	9	9	
	Approx. 5 mo	—	—	54	—	—	—	54	
Dead pups	Birth–1 mo	—	23	27	33	54	8	9	154
	1–3 mo	23	—	7	11	19	23	3	86
Adult females	Live (captures)	43	40	53	17	22	66	45	286
	Necropsy	2	3	1	4	3	2	1	16

standard diagnostic serology; in these cases, the enzyme-linked immunosorbent assay (ELISA) has been used (Sahly and Podschun, 1997). Although ELISA has been widely used to investigate the exposure of pinnipeds to some *Enterobacteriaceae*, such as *Salmonella* spp. and *Brucella* spp. (Aschfalk et al., 2002; Nielsen et al., 2005), the Western blot (WB) technique may represent an alternative to ELISA. The WB has shown comparable sensitivity and specificity for detection of antibodies against a variety of bacterial infections (Kittelberger et al., 1995). Unlike ELISA, the WB is not as dependent upon a negative serum panel for the calculation of a specific cut-off value. This is an advantage in wild pinnipeds where no such panel of known negative samples exists.

The main aim of this study was to determine prevalence estimates for antibodies to *K. pneumoniae* in the NZSL population on Enderby Island, before, during, and after 2 yr of epizootics caused by this pathogen. A secondary aim was to investigate passive transfer of humoral immunity to sea lion pups and to provide preliminary data on passive immunity in NZSL neonates.

MATERIALS AND METHODS

Collection of samples

Blood samples were collected from 240 NZSL pups and 16 adult females that were examined at necropsy at Sandy Bay Beach

rookery (Enderby Island) during the breeding seasons from 1997–98 to 2002–03, and in 2004–05. Blood was taken from the heart to avoid contamination when opening the carcass. Blood was centrifuged, and serum was frozen in liquid nitrogen. Serum also was obtained from live NZSLs: samples were collected from the caudal gluteal vein into sterile untreated glass tubes. Between 1997–98 and 2004–05, 286 adult females were captured for ongoing studies, and sampling was conducted under general anesthesia using isoflurane/O₂ (Gales and Mattlin, 1997). In May 2000, 54 pups of approximately 5 mo of age were manually restrained, and blood was collected as described above. In 2004–05, the same procedure was followed to collect blood samples in 20 pups varying in age from neonates to 2 mo old. Some individuals were sampled two or three times, resulting in the collection of 32 serum samples. For live pups, the age at sampling was known because these animals were identified and marked at birth with a plastic cap. For dead pups, the age could only be estimated based on the annual mean birth date (26 December; Chilvers et al., 2007) and the date of necropsy. Overall, the age of pups was categorized as follows: <1 mo, 1–3 mo, and approximately 5 mo (live pups captured in May 2000) (Table 1).

Preparation of antigens

Each *Klebsiella* isolate used in this study had been cultured from the internal organs of different pups, identified with standard systematic tests, and typed by pulsed-field gel electrophoresis (PFGE; Castinel et al., 2007b). Two *K. pneumoniae* isolates (one from 2001–02 [*K. pneumoniae* 1] and one from 2002–03 [*K. pneumoniae* 2]) and one *Klebsiella oxytoca* isolate (from a pup that was examined at necropsy in 2000–01) were used to prepare

antigens for the serologic test. Although PFGE results indicated that *K. pneumoniae* 1 and 2 were genetically indistinguishable (Castinel et al., 2007b), both were prepared as antigens to confirm clonality of their antigenicity. Previous PFGE results indicated that *K. oxytoca* was unrelated to the two other isolates (Castinel et al., 2007b). Antigens were prepared from the three *Klebsiella* isolates by mixing 0.5 g of washed bacterial pellet with 4.5 ml of polyacrylamide gel electrophoresis (PAGE) sample buffer (62 mM Tris-HCl, pH 6.9, 5% sodium dodecyl sulfate [SDS], 1% mercaptoethanol, and 10% glycerol), by heating at 95 C for 10 min, and finally by centrifuging at 16,700 × G for 15 min. The supernatants were used as antigens, and they were designated AG1 (*K. pneumoniae* 1), AG2 (*K. pneumoniae* 2), and AG3 (*K. oxytoca*). To further characterize antigens (lipopolysaccharides or proteins) and to optimize the WB technique, aliquots of the three crude antigens were incubated with proteinase K (pK, at 60 mAU/ml) at 56 C for 1 hr, and then they were analyzed by SDS-PAGE using Coomassie and silver staining methods as described by Kittelberger et al. (1994).

SDS-PAGE and WB

SDS-PAGE was performed on 4–15% polyacrylamide gradient gels (Ready-gels, Tris-HCl, Bio-Rad, Hercules, California, USA) with a discontinuous Tris-glycine buffer system in a Mini-Protean II electrophoresis cell (Bio-Rad). Gels were run for 40 min at 200 V, and then either used for WB or for gel staining (Kittelberger et al., 1994). Gels with 10 or 15 wells were used for the analysis of antigens for the WB optimization; preparative gels were used for the screening of serum samples for anti-*Klebsiella* antibodies (Klebs-WB). Electrophoretic transfer of separated antigens from polyacrylamide gels onto membranes (Immobilon P membrane, Millipore, Billerica, Massachusetts, USA) was carried out in a Trans-Blot SD cell (Bio-Rad) at 15 V for 20 min. Membranes were blocked overnight at room temperature (RT) in Tris-buffered saline (TBS) (50 mM Tris-HCl, 150 mM NaCl, and 0.01% merthiolate, pH 7.5) containing 3% nonfat dry milk powder (Anchor Ltd., Hamilton, New Zealand; TBS-MP). NZSL serum samples were diluted at 1:200 in TBS-MP and incubated at RT for 2 hr on the membranes in a miniblot system (Immunitics, Cambridge, Massachusetts, USA). The membranes were washed three times in TBS containing 0.05% Tween 20 (TBS-T), followed by incubation at RT for 30 min with protein A/G-alkaline phosphatase conjugate (Immunopure protein A/G, Pierce

Chemical, Rockford, Illinois, USA) diluted at 1:5,000 in TBS-MP. After three washes with TBS-T, the membranes were incubated with the substrate nitro blue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate (Roche Diagnostics, Mannheim, Germany) for 3 min at RT, followed by two washes with deionized water. Molecular weight (MW) markers (high-range rainbow MW markers, GE Healthcare, Chalfont St. Giles, UK) were run on every gel. In the Klebs-WBs, a protein A-purified rabbit anti-*K. pneumoniae* antibody (B65891R, 4–5 mg/ml, Biodesign International, Saco, Maine, USA) was run as positive control at dilutions 1:5,000, 1:20,000, and 1:80,000 in TBS-MP. Serum from a NZSL pup was run as a negative control at a 1:200 dilution in TBS-MP. The pup had died from traumatic injury before the epizootic years. None of its internal organs (lungs, liver, spleen, several lymph nodes, thymus, and feces) were positive on bacterial culture. This serum did not show any staining in the Klebs-WB. Intensity of staining in the Klebs-WB was coded as follows: negative=0, positive-weak (positive control 1:80,000)=1, positive-mild (positive control 1:20,000)=2, and positive-strong (positive control 1:5,000)=3.

Serum IgG concentrations

For the determination of serum IgG concentrations, a WB was developed (IgG-WB) based on a similar method used for bovine IgGs (Kittelberger, unpubl. data). Immunoglobulin G standards were prepared from NZSL adult female serum samples by ammonium sulfate precipitation. The IgG concentration was determined using the QuickStart protein assay (Bio-Rad). The purity of the IgG preparation from NZSL adult females was determined by SDS-PAGE and Coomassie staining. For the IgG-WB, serum samples were diluted in SDS-PAGE sample buffer at 1:10 for pups and 1:40 for older sea lions. Immunoglobulin G standards were run at concentrations of 1.6, 0.8, 0.4, 0.2, and 0.1 mg/ml (diluted in SDS-PAGE sample buffer) on every gel, along with serum samples to be tested. SDS-PAGE was performed on 15-well 4–15% polyacrylamide gradient gels, followed by electrophoretic transfer, blocking with TBS-MP, and incubation with conjugate and substrate, as described for the Klebs-WB. IgG concentrations were calculated from blot images using the Quantity One program (Bio-Rad).

Data analyses

For statistical analysis of the Klebs-WB results, five age classes of NZSLs were defined as follows: <1 mo (1), 1–2 mo (2), 2–3 mo (3),

approximately 5 mo (4), and adult females (5). The frequency of the four possible responses obtained with the Klebs-WB (0, 1, 2, and 3) was analyzed by year and by survival (dead=0 and alive=1) with the FREQ procedure in Statistical Analyses System® 9.1.3 (SAS, SAS Institute Inc., Cary, North Carolina, USA) separately for pups and for adults. The effect of year and survival on the variable “serologic test” was investigated with a generalized logits model (LOGISTIC procedure with GLOGIT option in SAS). Immunoglobulin G concentrations are reported graphically only, and medians were estimated for <8-wk-old pups, for 5-mo-old pups, and for adult females. When concentrations were too high to be calculated with the standard curve, the individual was given the maximum concentration allowed by the method (40 mg/ml for pups and 80 mg/ml for adult females). The same rule applied to concentrations beyond the lowest limit (1 mg/ml for pups and 2 mg/ml for adult females). Data are presented as means except for IgG concentrations as stated above. A statistical difference was assumed when $P < 0.05$.

RESULTS

Optimization of WB methods (Klebs-WB and IgG-WB)

For the detection of anti-*Klebsiella* serum antibodies by the Klebs-WB, three antigens—AG1, AG2, and AG3—were analyzed by SDS-PAGE using a combination of undigested and pK-digested antigen preparations and Coomassie staining (proteins) and silver staining (polysaccharide) (data not shown). In the Coomassie-stained gels, the undigested antigen preparations showed numerous distinct protein bands over a wide MW range (20–100 kDa). These protein bands disappeared completely on the Coomassie-stained gel after enzymatic digestion, but the silver-stained gels still showed strong staining for digested antigen preparations. The staining patterns were strong and diffuse over a wide MW range (20–200 kDa), which is typical for smooth lipopolysaccharides (SLPSs) (Tomás et al., 1986; Kittelberger et al., 1995). After running a commercial rabbit-anti-*Klebsiella* serum and a number of NZSL samples in the Klebs-WB, it became obvious that SLPS was immunodominant

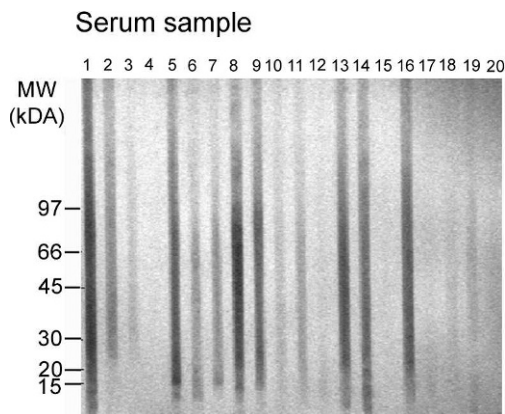


FIGURE 1. Example of an optimized Western blot for the detection of antibodies to *Klebsiella* spp. in New Zealand sea lions (NZSLs). Each lane represents a single serum sample. Lanes 1 to 3 are dilutions of a rabbit-anti-*Klebsiella* control serum. Lane 4 is the negative control. Lanes 5 to 20 are NZSL sera. As an example, lane 5 is positive-strong (grade 3), lane 6 is positive-mild (grade 2), lane 10 is positive-weak (grade 1), and lane 15 is negative (grade 0).

and that the three antigen preparations were immunologically identical. Therefore, the pK-digested AG1 was used for this study. Staining patterns were classified as strong, medium, weak, and negative. The rabbit anti-*Klebsiella* control antibody had an IgG concentration of 4–5 mg/ml. As an approximation and assuming that the majority of IgG in the positive control serum was anti-*Klebsiella* IgGs, strong staining corresponded to 0.8–1 $\mu\text{g/ml}$ anti-*Klebsiella* IgGs, medium staining to 0.2–0.25 $\mu\text{g/ml}$, and weak staining to 0.05–0.06 $\mu\text{g/ml}$ specific IgGs. NZSL serum samples that showed at least weak staining in the Klebs-WB were considered positive for anti-*Klebsiella* antibodies. Conditions for the detection of total IgGs by the IgG-WB were also optimized by testing various concentrations of sea lion sera and conjugate (data not shown).

Klebs-WB results

An example of a Klebs-WB of 12 NZSL sera is presented in Figure 1. Frequency of NZSLs that tested positive for circulating anti-*Klebsiella* antibodies from 1997–

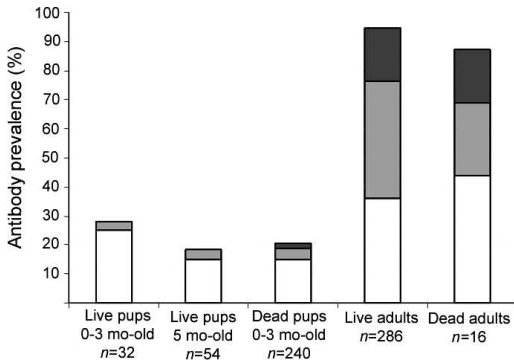


FIGURE 2. Distribution of the intensity of positive responses of New Zealand sea lion (NZSL) adult females and pups tested for anti-*Klebsiella* serum antibodies, from 1997–98 to 2004–05. The proportion of intensity of the positive reaction between NZSL sera and the test antigen are presented by grades: weak (white), mild (grey), and strong (black).

98 to 2004–05 is shown for all animals by age categories in Figure 2, and for pups only by year in Table 2. Specific antibodies were detected throughout the study, but overall, only 16% of NZSL pups between birth and 5 mo of age were seropositive compared with 95.7% of adults. Neither year nor survival had a significant effect on the Klebs-WB response in pups and in adult females. Although there was a higher prevalence of pups possessing anti-*Klebsiella* antibodies in 1998–99 (39%) and in 2004–05 (28%) compared with the other years (Table 2), these differences were not statistically significant. In contrast, the age of pups

significantly influenced the presence of such antibodies ($P < 0.0001$).

Amongst seropositive pups, very few showed a strong staining on the Klebs-WB (Fig. 2), and the majority of staining was weak. The prevalence of anti-*Klebsiella* antibodies did not vary between seasons for adult females at necropsy, but it was significantly different when considering the overall population of adult females ($n = 302$, $P < 0.0001$) or the subpopulation of live adults only ($n = 286$, $P < 0.0001$). Prevalence of the presence of anti-*Klebsiella* antibodies in adults ranged from 86% in 1997–98 to 100% in 2000–01 and 2001–02 (Table 2).

IgG serum concentrations

In the IgG-WB, the IgG band had an approximate MW of 130 kDa. The absolute concentrations of IgGs detectable by this method ranged from 0.1 mg/ml to 1.6 mg/ml. Because samples had to be prediluted, the method covered a range from 1 mg/ml to 80 mg/ml for the detection of total IgG in serum samples. The IgG concentrations for 1–2-wk-old pups ranged from <1 mg/ml to 10 mg/ml, whereas values in older pups were more skewed toward higher concentrations, suggesting that serum IgG levels increased with age in this group. Immunoglobulin G concentrations ranged from <1 mg/ml to >8 mg/ml in pups <8 wk old ($n = 32$, median 2.1 mg/ml), from 5.4 mg/ml to 40.0 mg/ml in pups of approximately 5 mo of age ($n = 8$, median

TABLE 2. Prevalence of New Zealand sea lion pups and adults (live and dead) with anti-*Klebsiella* serum antibodies from 1998–99 to 2004–05.

Age		Yr					
		1998–99	1999–2000	2000–01	2001–02	2002–03	2004–05
Pups	Dead	9/23 ^a (39)	6/34 (18)	5/44 (11)	5/73 (7)	1/30 (3)	3/12 (25)
	Live	—	10/54 (19)	—	—	—	9/31 (29)
	Total	9/23 (39)	16/88 (18)	5/44 (11)	5/73 (7)	1/30 (3)	12/43 (28)
Adults	Dead	2/3 (67)	1/1 (100)	4/4 (100)	3/3 (100)	1/2 (50)	1/1 (100)
	Live	40/40 (100)	50/52 (98)	13/13 (100)	22/22 (100)	62/64 (97)	41/44 (93)
	Total	42/42 (98)	51/53 (98)	17/17 (100)	25/25 (100)	63/66 (96)	42/45 (93)

^a Number of animals possessing anti-*Klebsiella* antibodies/total number of animals tested (percentage).

22.0 mg/ml), and from 2.0 mg/ml to >80.0 mg/ml in adult females ($n=33$, median >80.0 mg/ml). Although the limited number of serum samples precluded a reliable statistical comparison between pups and adults, apparent differences were observed between the two groups (IgG concentrations were higher in adults than pups) and within pups (older pups had higher IgG levels than newborns).

DISCUSSION

An SLPS-WB was developed to explore the seroprevalence of anti-*K. pneumoniae* antibodies in NZSL pup and adult sera before, during, and after two consecutive epizootics caused by the pathogen. According to the Klebs-WB results, a small number of pup sera and almost all adult sera were seropositive, without any significant change in prevalence during both epizootics. Despite this high antibody prevalence in adults, low IgG levels were present in pup sera, suggesting that pups could be highly vulnerable to this bacterial pathogen during the first weeks of life.

After optimization with pK digestion, the Klebs-WB not only identified seropositive and seronegative sea lions but also showed that the majority of serum antibodies were directed against *Klebsiella* SLPS. However, this serologic technique could not discriminate between serum antibodies against the immunologically related *K. pneumoniae* and *K. oxytoca* (Rissing et al., 1978; Sechter et al., 2000). This lack of specificity did not negatively impact the present study because the WB technique proved to be a suitable method for screening large numbers of archived serum samples and seemed to be very sensitive. Klebs-WB analyses showed that anti-*Klebsiella* antibodies were present in all age groups before, during, and after the *K. pneumoniae* epizootics. Such antibodies were detected before the epizootics, even when *Klebsiella* species had not been isolated from tissues of NZSLs from Sandy Bay Beach or from animals (associated

with accidental bycatch during squid fishing) sampled around the Auckland Islands (Castinel, unpubl. data). Although the prevalence of pups testing positive for anti-*Klebsiella* antibodies was significantly lower than for adults, these antibodies were present in pups before the first *K. pneumoniae* epizootic in 2001–02.

The prevalence of anti-*Klebsiella* antibodies (most of which were detected by medium and strong staining in the Klebs-WB) were very high for adult females compared with 1–2-wk-old pups. Antibodies in pups were limited to pups >7 wk of age, suggesting that seroconversion would take a minimum of approximately 2 mo after exposure. This also suggested that maternal antibodies were not passively transferred to newborns, or that they existed at a level that was not detectable; the Klebs-WB sensitivity limit was 0.8 mg/mg. Only 19% of live pups (ca. 5 mo old) were seropositive in May 2000 compared with 100% of pups (8 wk old) sampled in 2004–05. This difference probably relates to the time that samples were collected rather than age. The 2000 and 2004–05 samples were taken before and after the outbreak, respectively.

To investigate passive immune transfer in NZSLs, a WB test was developed to quantify total IgGs in pups, at least in a limited number of serum samples. Although numerous sandwich ELISAs are commercially available for the determination of immunoglobulin levels in a number of domestic animal species, no such ELISA existed for NZSL immunoglobulins. The IgG-WB method used in the present study was able to detect serum IgG levels as low as 1 mg/ml. In 1–2-wk-old pups, IgG levels were all <10 mg/ml, whereas in adults concentrations of >20 mg/ml were observed. Serum IgG levels in pups were consistent with those reported in gray seal (*Halichoerus grypus*) pups of approximately the same age (Carter et al., 1990), but higher values were reported in northern fur seal (*Callorhinus ursinus*) pups (Cavagnolo and Vedros, 1979). Additional data from

NZSL pups is needed to statistically compare IgG levels with other otariid species.

The presence of *K. pneumoniae* on the rookery early in the breeding season was confirmed by bacterial cultures from tissue samples collected at necropsy. Based on isolation data, variation in the extent of potential exposure to *K. pneumoniae* varied over the eight consecutive breeding seasons at Sandy Bay Beach (Castinel et al., 2007a,b). There are still many unknown factors surrounding the *K. pneumoniae* epizootics at Sandy Bay Beach rookery, especially regarding the conditions of introduction of the pathogen in the colony. Although the Klebs-WB analyses demonstrated that anti-*Klebsiella* antibodies were present in all NZSL age groups before, during, and after the *K. pneumoniae* epizootics, results did not provide definitive evidence to indicate that *K. pneumoniae* was enzootic in the NZSL population or whether it had been introduced on the rookery in 2001–02.

Although there was some evidence of a humoral immune response directed against *Klebsiella* species before the epizootics, this previous exposure did not seem to be protective against the *K. pneumoniae* epizootic strain. This could be explained by the high virulence of the *K. pneumoniae* strain, an ineffective or inadequate level of herd immunity before the outbreak, and a low level or nonprotective passive transfer of anti-*Klebsiella* antibodies to pups.

ACKNOWLEDGMENTS

The authors thank W. Hockley, S. Childerhouse, N. Gibbs, H. Best, J. Amey, F. Riet-Sapriza, M. Brown, and R. Hood for assisting with collection of blood samples in the field, R. Pattison for preparing the antigen, and all the staff and administration at the Investigation and Diagnostic Centre-Wallaceville for offering to conduct the serologic analyses within personal laboratory facilities. The authors are also very grateful to S. C. Tolliver, E. T. Lyons, and C. Pigott for comments. Approval for this work was obtained from the New Zealand Department of Conservation (DOC), and the

research was conducted under permits from DOC and Massey University Animal Ethics Committees. This study was partly funded by the Institute of Veterinary, Animal and Biomedical Sciences (IVABS, Massey University, Palmerston North, New Zealand), by the IVABS Lewis Fitch Fund, by the Joan Berry Fellowships in Veterinary Science Scholarship and by DOC.

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Received for publication 13 March 2007.