Assessing Beaufort Sea Polar Bear Health: Associations between hematological and serological endpoints

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ABSTRACT
A study of free ranging populations of polar bears in northern Alaska has been initiated to establish clinical (health) baseline data in order to monitor potential change in health status using multiple hematologic endpoints and infectious agents exposure measures. Plasma protein, hematocrit, total leukocyte and leukocyte differential counts measured fall within one standard deviation of values reported for captive polar bears (International Species Database). A relatively high prevalence of serum antibodies to four morbilliviral species [canine distemper (CDV), dolphin morbillivirus (DMV), phocine distemper (PDV), and porpoise morbillivirus (PMV)] were also identified. This group of viruses can cause significant disease and mortality in populations of some marine mammals as well as interfere with differentiation and specialization of lymphocytes *in vitro*. Serological data indicate 49% of animals tested (n=137) in 2005 and 2006 combined were positive for antibodies to CDV, DMV, PDV, and/or PMV via differential serum neutralization. Antibodies to *Toxoplasma gondii* were found in 13% and 12% of animals tested for consecutive sample years, with titers as high as 1:2048. Relationships between antibody titers and hematological parameters were examined, revealing significant decreases in total leukocytes, neutrophils, monocytes and eosinophils with increasing CDV titer. In addition, a significant positive relationship was found between eosinophils and increasing titer to *Toxoplasma gondii*. Functional assays (e.g. blastogenesis), further sample collection (Spring 2007) and pending analyses including an epidemiologic approach will assist in evaluating the biological significance of these observed changes.

KEYWORDS

ARCTIC, DISEASE, EPIZOOTIC, MONITORING, CLIMATE CHANGE

INTRODUCTION

Studies in Svalbard (Norway) and northern Canada (Bernhoft et al., 1997; Brunstrom and Halldin 2000, Chiu et al., 2000; Norstrom et al., 1998; Skaare et al., 2000) suggest that contaminants and climate change likely constitute the two greatest anthropogenic stressors to the health of polar bear populations. Based on these and other studies, there is concern that such stressors may have resulted in an immune and reproductively compromised population of bears that may be more vulnerable to disease and have reduced life expectancy and reproductive performance (Wiig et al. 1998). In the spring of 2005, a study was initiated to increase baseline health data collected for the southern Beaufort Sea population of polar bears in northern Alaska. The goal of these efforts was to facilitate health and immune status monitoring in the face of increasing anthropogenic stressors. The study employed multiple hematologic endpoints including; plasma protein, hematocrit, total leukocyte and leukocyte differential counts. Specific antibody assays included serum neutralization for morbilliviruses and latex agglutination for antibodies to *Toxoplasma gondii*. We chose to measure morbilliviral antibodies as this group of viruses can cause significant disease and mortality in populations of some marine mammals (Saliki et al. 2002) as well as interfere with and specialization and differentiation of lymphocytes *in vitro* (Heaney et al., 2002). Furthermore, immune suppression associated with morbillivirus infections may influence the mortality rate of infected individuals by allowing secondary bacterial (or other pathogens) infections that are lethal to the host.

Some of these agents have been documented to occur in Alaska polar bears (Follmann et al., 1996; Garner et al., 2000), Alaska and Canada brown bears (Chomel et al., 1998; Philippa et al., 2004) and arctic foxes (Ballard et al., 2001); and have significant potential for an epizootic in pinnipeds (e.g. De Koeijer et al., 1998; Hall et al., 1992; and others). Polar bears seropositive for CDV-like viruses have been found in other regions of the Arctic including Canada and Svalbard where prevalences have been documented at 24% and 8%, respectively (Cattet et al. 2004; Tryland et al. 2005).

MATERIALS AND METHODS

Location and animal capture

Blood samples were taken from polar bears captured in the Alaskan Beaufort Sea spanning a six week period from March through May in the springs of 2005 and 2006. Bears were captured by injection of Telazol® administered by darts fired from helicopters. Bears were captured out of three locations along the North Slope of Alaska including: Barrow, Prudhoe Bay, and Barter Island.

Blood collection and processing

Blood was drawn into evacuated test tubes (BD Biosciences) by venipuncture of the femoral vein (occasionally the artery). Sera derived from blood without anticoagulant were separated by centrifugation at 3,500 rpm for 5 minutes and frozen at -20°C (TRIAC, Clay Adams Co., Parisippany, N.J.). Sera were stored at -70°C upon return from the field for later serological assays. Blood collected into EDTA treated tubes was used to prepare slides for leukocyte differentials and for the determination of total hematocrit, plasma protein and leukocyte counts. These endpoints were determined on the day of capture to avoid time dependent post sampling changes.

Hematology

Total leukocyte counts were determined microscopically using the Unopette® system and a hemocytometer on the day of capture. Differential blood cell counts were conducted on blood smears stained with Wright's-Giemsa type stain by identifying and classifying 100 leukocytes into the specific morphologic types (neutrophils, monocytes, eosinophils, and basophils). In 2005, blood smears were fixed in the field and stained within1month. In 2006, we optimized the smears by fixing and staining on the date of sample collection which prevented the cells from over-drying. This decision was made based on the presence of staining artifact in 2005 which obscured nuclear granules. Three independent readers conducted differential counts and statistical bias was assessed by plotting values obtained by readers against one another, using the most experienced reader's value as the independent variable. Counts were statistically unbiased among readers and therefore count values obtained for all readers were averaged for each individual animal. Absolute counts were also determined by finding the product of mean percentage and total white blood cell count per individual. Total hematocrit was determined for whole blood treated with EDTA (anticoagulant) using a high-speed micro centrifuge for 3 minutes at 10,400 rpm (TRIAC, Clay Adams Co., Parisippany, N.J.), by measuring packed cells as a percent of blood volume. Total plasma protein was determined using hand-held refractometer (SPER Scientific 300005). Hematological reference ranges were taken from the International Species Database (ISIS) which catalogues physiological data collected from captive polar bears and other wildlife species as field data of this type has not been established for polar bears. Reported ranges were based on approximately 200 samples collected from 90 individual polar bears within 34 institutions worldwide.

Serology

Serological assays for morbillivirus and *Toxoplasma gondii* were performed at Oklahoma State University Animal Disease Diagnostic Laboratory. A differential serum neutralization (SN) assay was performed using four morbilliviruses [canine distemper (CDV), dolphin morbillivirus (DMV), phocine distemper (PDV), and porpoise morbillivirus (PMV)] as described by Garner et al. (2000). Results were expressed as reciprocal of the highest dilution that completely neutralized 100% of the respective challenging virus ('neutralized'). A latex agglutination assay was performed for *Toxoplasma gondii*. Results are expressed as the reciprocal of the highest dilution of serum that resulted in a clear agglutination.

Statistics

Summary statistics were calculated for all parameters and normality assessed using boxplots and the Shapiro-Wilk goodness of fit test; log transformations were employed where necessary. Hematologic endpoints were cast in a correlation matrix for sample years 2005 and 2006 independently as well as combined, in order to assess bivariate relationships (JMP, SAS Institute). Pearson's correlations were taken to be significant where $p \leq 0.05$. For parameter pairs whose correlations were significant, percentage mean difference values were determined by calculating the difference between the mean of a given variable for all CDV positive and negative individuals and dividing this number by the mean for seronegative animals.

RESULTS

Plasma protein, hematocrit, total leukocyte and leukocyte differential counts measured in the springs of 2005 (n=63) and 2006 (n=68), are within one standard deviation of values reported for captive polar bears (ISIS). Relatively high prevalences of serum antibodies to four morbilliviral species CDV, DMV, PDV, and PMV were identified in both capture years 2005 and 2006. The most striking result was 48% and 50% prevalence of CDV antibodies for consecutive capture years. In addition, each animal that was positive for either DMV, PDV, or PMV antibody presented a higher titer for CDV (see figure 1 and table 1). 13% and 12% of animals tested positive for antibodies to *Toxoplasma gondii* for sample years 2005 and 2006 respectively

Relationships between antibody titers and hematological parameters were examined, revealing significant decreases in neutrophil, eosinophil, monocyte, and total leukocyte counts with increasing CDV titer (see table 2). Relationships were statistically significant when data were analyzed for 2005 as well as 2005 and 2006 combined; however no significant associations were found when 2006 data was analyzed alone. When only seropositive animals are considered for combined capture years, subadults displayed a significantly higher mean titer for CDV antibodies as compared to adults. No differences in titers were observed between genders. In addition, a significant positive relationship was found between the number of eosinophils counted and sero-titer for 2005 bears. Eosinophils are a type of white blood cell released in response to parasitic infections and allergens. This finding, together with some of the high titers observed, is suggestive of an active infection rather than a previous infection or exposure. The same relationship was not found to be significant for 2006 data alone. Mean titers for animals seropositive for *Toxoplasma gondii* were not significantly different between capture years.

DISCUSSION

The high prevalence of morbilliviral antibodies (especially CDV) is consistent across sample years and with previous studies conducted in Alaska. That each individual positive for either PDV, DMV, or DMV presented a higher titer to CDV suggests that the virus(es) to which northern Alaskan polar bears have been exposed is most antigenically related to CDV. Cross-reactivity among these antibodies has previously been demonstrated (Saliki et al. 2002). These findings are consistent with that of Garner et al. (1999) who concluded that the virus present in polar bears was most likely of terrestrial origin.

The observed changes in hematological parameters (TWBC, neutrophil count, eosinophil count, and monocytes count) are suggestive of a biologically significant effect of pathogen exposure. The presence of statistically significant negative associations between these parameters and increasing CDV titer suggests that the virus may have an immunosuppressive effect. That statistical significance was achieved in 2005 alone, but not 2006 may reflect natural fluctuation in the population in response to changes in pathogen exposure and/or immunity. Alternatively, the effect may be the result of an artifact due to limited power in the 2006 data set (e.g. greater variance). That subadults demonstrate a statistically greater mean titer as compared adults may indicate initial protection via maternal antibodies followed by a spike in titers at sexually maturity when bears become independent and begin interacting with one another. This hypothesis is supported by an examination of the percentage of seropositive bears at each age (see figure 3). Further analyses are currently pending, including an epidemiologic approach.

While significant hematological associations in Beaufort Sea polar bears have been observed, we do not know if seropositive animals are adversely affected by and/or susceptible to morbilliviral infection. No overt clinical signs typically associated with the disease in domestic or wildlife species were observed upon gross examination of captured animals and no associations between body condition score and seropositivity were detected. The presence of antibodies in these animals may be the result of exposure only, with no significant resulting viremia. In an alternate scenario, the virus may be able to replicate within polar bears without any adverse effects, yet they may serve as a reservoir for the disease for other arctic carnivores.

Over the past two decades, this group of four closely related morbilliviruses has been documented to cause significant disease and mortality in several marine mammal species, such as phocine distemper (PDV) in harbor seals (*Phoca vitulina*) in the North Sea in1988 (Dietz et al. 1989) and dolphin morbillivirus (DMV) in striped dolphins (*Stenelle coeruleoalba*) in the Mediterranean in 1990 (Aguilar and Raga, 1993). CDV was also demonstrated to be the primary cause of mortality in Caspian seals in a 2000 epizootic (Kennedy et al., 2000). In all mortality events documented among seals and dolphins attributed to morbilliviral infections, these top predators were also exposed to high levels of persistent lipophilic environmental contaminants accumulated through the food chain (de Swart et al. 1996). This observation led to the hypothesis that contaminant related immunosuppression may have contributed to the severity of the outbreaks.

Contaminants reach the Arctic via long range atmospheric transport and via ocean and river currents from lower latitudes. Apex predators within the Arctic, such as the polar bear (*Ursus maritimus*) have been demonstrated to carry the highest burdens of some of these chemicals. The polar bear resides at the top of the arctic food web, which contains high lipid levels in biota with many trophic levels, thus this species tends to bioaccumulate lipophilic compounds such as OCs which are by definition resistant to physical and biochemical degradation. The effects of contaminants on polar bears are of particular concern as a result of some unique physiologic features which may render them more vulnerable to the adverse effects of OCs. These features include dramatic periods of fat accumulation followed by extended periods of fasting which can affect the internal dynamics of OCs within their systems (shared with some other arctic species, e.g.; ringed, fur, spotted, and ringed seals); delayed implantation and unique mechanisms of biotransformation (an effective cytochrome P450 system), which result in accumulation of different mixes of contaminants as compared to other species.

Many OCs disrupt both humoral and cell-mediated immune responses of the specific (acquired) arm of the immune system, as well as causing effects in the non-specific (innate) arm. Resistance to infectious agents may be reduced as a result. Humoral-mediated immunity involves the body's ability to recognize foreign substances (via helper T-cells) and mount a response by stimulating the production of antibodies (from B-cells). Cell-mediated immunity is involved in delayed hypersensitivity (e.g. skin reactions and the production of cytotoxic T-cells against tumors and viruses. Natural killer cells are involved in the non-specific immune response and provide the first line of defense against virus-infected cells. Immunosuppressive effects have been documented in harbor seals (*Phoca vitulina*) fed Baltic fish in semi-field experiments and was found to correlate with levels of polychlorinated dibenzo-p-dioxins (PCDD), polychlorinated dibenzo-furans (PCDFs) and planar PCBs (expressed at TEQs) (De Swart et al., 1995; Ross et al., 1995, 1996). In addition, two studies on polar bears at Svalbard demonstrated negative correlations between PCBs and antibody-mediated immunity (Bernhoft et al., 2000, Lie et al., 2002). With potential changes in contaminant dynamics due to climate change, the levels of contaminants in polar bears may also change. When combined with other stressors, such as malnutrition and disease, a synergistic effect may occur in which one factor is aggravated by another or others.

In canines, and some wildlife species, canine distemper infection is characterized by immunosuppression followed by secondary infections. For example, Moro et al. (2003) demonstrated increased apoptosis in lymphoid tissue of naturally and experimentally infected dogs as compared to uninfected controls. Lymphopenia was the most

important hematological alteration observed. Another *in vitro* study demonstrated that infection with CDV results in both virus-dependent and independent immunosuppression. In this co-culture study, lymphocytes suppressed the phytomitogen responses of uninfected responder dogs. In addition, lymphocytes from nonviremic dogs also suppressed the responses of uninfected responder dogs (Krakowa 1982). In a 1987 study, Krakowa et al. (1982) demonstrated that in short term micro-cultures, CDV infection of canine mononuclear cells resulted in suppression of lectin induced 3H-thymidine incorporation. Suppression of pokeweed mitogen-driven *in vitro* immunoglobulin synthesis and release was also observed. Finally, interleukin-1 production by adherent mononuclear cells was significantly depressed and monocyte cultures derived from viremic dogs released prostaglandin (PG) E2. These results suggest that, not only can CDV directly effect lectin responsive cellular population(s), but the virus can also modulate monocyte functions by inhibition of interleukin-1 production and by enhancing PGE2 release (Krakowa et al., 1987).

Infectious disease affects the population dynamics of large carnivores and abundance of animals, however the potential role of disease in wildlife conservation has only recently drawn considerable attention (e.g. Dobson and May, 1986; May, 1988; Scott, 1988; Thorne and Williams, 1988; MacDonald, 1993; Murray et al. 1999). These findings suggesting potential immunosuppressive effects are particularly important given that critical foraging habitat for polar bears appears to be decreasing with shrinking summer ice cover. This issue is important for many ice dependent species and the associated pathogens. For example, studies conducted by the USGS for Beaufort Sea polar bears have documented decreased survivorship among cubs of the year and smaller skull size of adult males for the period 1990-2006 in comparison to animals studied prior to1990. (U.S. Geological Survey, Open-File Report 2006-1337). In addition, predation events by adult males upon denning females have been recently observed. The authors of this study speculate that this atypical phenomenon may be an indication of a nutritionally stressed population (Amstrup et al., 2006). The effects of any given disease within a population may be aggravated if they interact with factors such as malnutrition, stress, or exposure to contaminants.

CONCLUSION

The ecology of morbillivirus on the North Slope of Alaska requires further study. Based on serological assays it cannot be determined whether these bears are infected or exposed to multiple morbilliviruses or perhaps carry a distinct strain endemic to polar bears in Alaska and/or the Arctic. If viral nucleic acid can be isolated from polar bears, sequence data can provide valuable information regarding probable sources and transmission pathways. To date, morbilliviral RNA has never been isolated from any ursid species; therefore little is known regarding the phylogenetic relationship of these strains to those strains carried by compatric carnivore species. Serosurveys of other arctic wildlife [e.g. arctic fox (*Alopex lagopus*)] and domestic dogs (*Canis familiaris*); as well as polar bear prey (ice seals) would also assist in the elucidation of the ecology of morbilliviral species in Alaska.

In vitro studies using lymphocytes harvested from polar bears can be used to move beyond statistical associations. For example, blastogenesis can be compared between seropositive and seronegative animals to search for a mechanistic and quantifiable difference in immune capability. The results of this study underscore the importance of efforts to monitor Beaufort Sea polar bears on a population as well as physiological level. The availability of baseline health data on these animals can provide valuable information in the face of increasing stressors and may have predictive (prognostic) value. Special consideration should be given to synergism among stressors faced by this species which may compound adverse effects thereby undermining resilience.

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FIGURES AND TABLES

Fig. 1 Cell type counts for polar bears captured in 2005 and 2006 (mean ±1 SD).

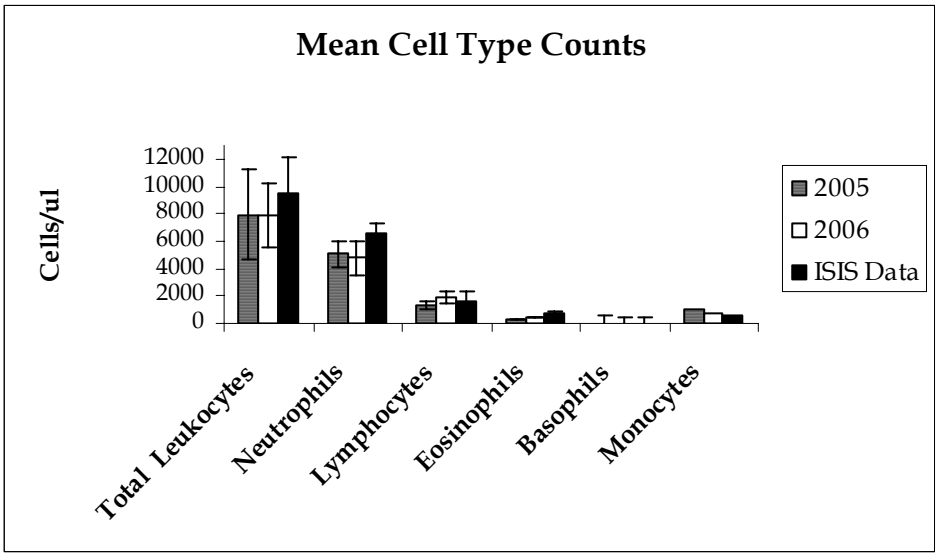

Table 1. Antibody Prevalence Rates for polar bears captured in 2005 and 2006.

Antibody Prevalence Rates:	2005 (n=64)	2006 (n=73)	2005+2006 (n=137)
Canine Distemper Virus (CDV)	48% (31)	51% (37)	50% (68)
Phocine Distemper Virus (PDV)	20% (13)	27% (20)	24% (33)
Dolphin Morbillivirus (DMV)	6% (4)	7% (2)	6% (6)
Porpoise Morbillivirus (PMV)	2% (1)	3% (5)	6% (6)
Toxoplasma gondii	13% (8)	12% (10)	13% (18)

Figure 2. Titers for polar bears seropositive in morbillivirus serum neutralization differential (1/mean ± SE).

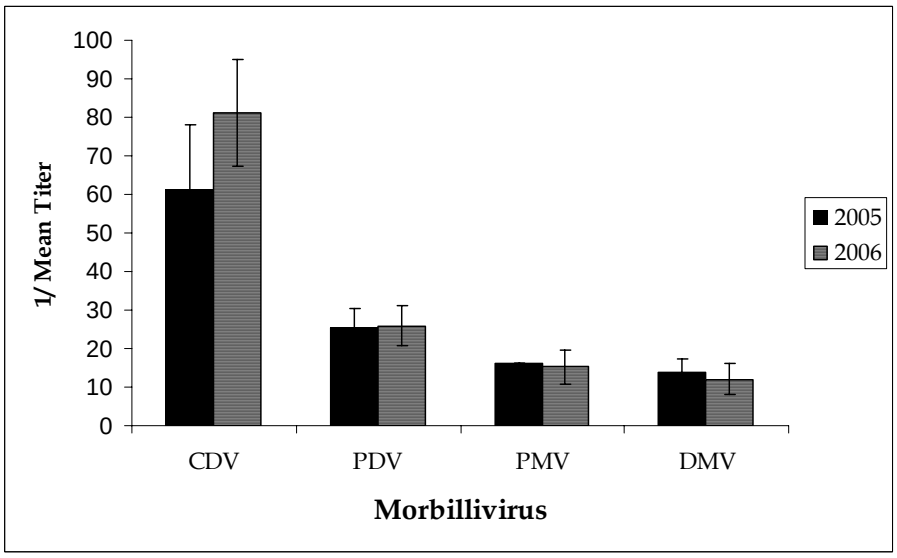

Table 2. Correlations between hematological and serological parameters.

		2005 (n=61)			2006 (n=67)		2005 + 2006 (n=128)		
Variable	**By Variable**	**rho**	**p**	**MD**	**rho**	**p**	**rho**	**p**	**MD**
CDV titer	TWBC	-0.36	0.00	-26.10%	-0.22	0.08	-0.28	0.00	-22.6%
CDV titer	Neutrophils	-0.27	0.03	-28.21%	-0.11	0.36	-0.19	0.03	-23.9%
CDV titer	Eosinophils	-0.31	0.01	-50.4%	-0.11	0.41	-0.18	0.04	-29.9%
CDV titer	Monocytes	-0.27	0.03	-33.1%	-0.16	0.22	-0.22	0.01	-27.8%

Variables were log transformed before correlations calculated. MD= mean difference.

Figure 3. Percentage of polar bears CDV positive at each age in 2005 + 2006.