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Assay dependence of *Brucella* antibody prevalence in a declining Alaskan harbor seal (*Phoca vitulina*) population

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Abstract

Background: *Brucella* is a group of bacteria that causes brucellosis, which can affect population health and reproductive success in many marine mammals. We investigated the serological prevalence of antibodies against *Brucella* bacteria in a declining harbor seal population in Glacier Bay National Park, Alaska.

Results: Prevalence ranged from 16 to 74 percent for those tests detecting antibodies, indicating that harbor seals in Glacier Bay have been exposed to *Brucella* bacteria. However, the actual level of serological prevalence could not be determined because results were strongly assay-dependent.

Conclusions: This study reinforces the need to carefully consider assay choice when comparing different studies on the prevalence of anti-*Brucella* antibodies in pinnipeds and further highlights the need for species- or taxon-specific assay validation for both pathogen and host species.

Keywords: *Brucella*, Harbor seals, Alaska, Assay dependence

Background

Harbor seal (*Phoca vitulina*) populations serve many important biological and sociocultural functions in Alaska. Harbor seals are apex predators and serve as an important prey for species such as killer whales (*Orcinus orca*) and Steller sea lions (*Eumetopias jubatus*) [1-3]. Harbor seals are also important subsistence food for many local communities, and play a prominent role in the culture of the Huna-Tlingit and other tribes. Glacier Bay National Park (GBNP) supports a unique stock of harbor seals in Alaska [4] and historically was the location of the largest breeding population of seals in the state. Yet the population has been declining since 1992 [5] even though foraging and other conditions have supported significant increases in other marine mammals, such as Steller sea lions and humpback whales, that utilize the park during the summer [6,7]. Hypotheses for the causal factors driving the decline include anthropogenic disturbance, although a recent study demonstrated that disturbance is not likely to be sufficiently frequent to

influence population dynamics [8]. Large populations of forage fish persist in GBNP [9], making food availability unlikely as a possible explanation for the population decline, and seals that breed in Glacier Bay are of similar physiological condition as those in other stable or increasing populations [10]. Predation by the increasing population of Steller sea lions has also been proposed although this is not likely to be the sole factor driving population dynamics [3]. Together then a number of hypotheses (food availability, disturbance, predation) have been addressed with no significant evidence identifying the cause for the observed decline in this population.

Here we perform a serological survey for exposure to *Brucella* bacteria, pathogens of concern in marine mammals in Alaska. In a recent study we determined the prevalence of antibodies against *Leptospira* spp., *Toxoplasma gondii* and distemper viruses as well as presence of *Giardia* and *Cryptosporidium* in fecal samples from this population [11] but we did not investigate the presence of *Brucella* bacteria, which are known to be present in harbor seals in Southeast Alaska [12]. *Brucella* bacteria have been identified as pathogens in marine mammals since 1994 [13] and have since been isolated, or anti-*Brucella* antibodies have been detected, in multiple

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marine mammal species throughout the world [14,15]. Brucellosis is a bacterial infection that can affect reproductive organs and therefore influence fecundity and lead to reduced recruitment and alter population dynamics [15], although in pinnipeds overt pathological findings have so far not been observed [14]. In addition, marine-derived *Brucella* bacteria have significant zoonotic potential in people exposed to marine mammals [16-18].

In this study we tested serum samples described previously [11] for anti-*Brucella* antibodies using six different tests in order to gain insight into the serological prevalence to assess the possible exposure to *Brucella* bacteria in Glacier Bay harbor seals.

Methods

Samples

In order to determine if the harbor seal population in GBNP had been exposed to *Brucella* bacteria we performed a number of serological tests to detect anti-*Brucella* antibodies in seals captured in 2007 (49 animals) and 2008 (44 animals) from harbor seals in Johns Hopkins Inlet (58.84N 137.11W), Glacier Bay National Park (GBNP), Alaska as described previously [11]. Age classes were determined [19], and 46 animals were classified as pups, 19 as yearlings, 10 as subadults, and 18 as adults. 51 animals were female and 42 were male.

All animal sampling was in accordance with approval of Institutional Animal Care and Use Committees at the University of Alaska Fairbanks (protocol 07-37) and the State of Alaska Department of Fish and Game (protocol 07-16), as well as a permit from the National Oceanographic and Atmospheric Administration under the Marine Mammal Protection Act (Permit 358-1787-00).

Serological tests

Brucellosis card test

Brucellosis Card tests (Becton Dickinson, Cockeysville, MD, US) using *B. abortus* strain 1119-3 as the antigen was performed independently according to manufacturer's instructions at the University of Alaska Fairbanks and the diagnostic laboratory of Colorado State University to ensure consistency between operators.

B. abortus plate test

Harbor seal serum was pipetted onto etched glass plates. Standard *B. abortus* Standard Plate Antigen (Strain 1119-3, National Veterinary Services Laboratories, Ames, Iowa, US) was added and thoroughly mixed with the serum and the plate rotated and incubated for 8 minutes further rotated before agglutination was assessed in indirect light over a black background.

Competitive ELISA

Competitive ELISA was performed at Mystic Aquarium. This test uses an antigen derived from *Brucella* isolated from a harbor seal. Serum samples were tested at a 1:10 dilution and less than 25% inhibition was considered negative. 25-29.9% inhibition was classified as a suspect test and sera showing 30% or higher inhibition were classified as positive for antibodies to "marine" *Brucella* spp. [20].

B. ovis ELISA as well as B. canis RSAT

B. ovis ELISA as well as *B. canis* RSAT were performed at the diagnostic laboratory of Colorado State University. The *B. ovis* ELISA followed the NVSL SeroPro protocol using the REO198 Antigen. The *B. canis* RSAT test utilized a commercially available test kit (D-TEC®, Synbiotics, Kansas City, MO).

Statistical analysis

The 95% confidence intervals for serological prevalence were calculated as previously described [21]. The different tests were compared by calculating positive percentage agreement, negative percent agreement and the overall percent agreement as well as McNemar's chi square test for pair-wise comparison of the diagnostic assays used in this study.

Results

Using an ELISA assay detecting *B. ovis* antibodies and a rapid slide agglutination test (RSAT) detecting *B. canis* antibodies, we did not detect an antibody positive sample in 93 animals tested. A Plate test for anti-*Brucella* antibodies yielded a 74% (95% CI = 64-82%) serological prevalence rate. The commercially available card test used detected antibodies against *B. abortus* in 17% (95% CI = 10-26%) and 16% (95% CI = 9-25%) samples for UAF and CSU, respectively. To confirm these results we performed this test independently and obtained very similar results with 95% overall agreement. The competitive ELISA based on a marine *Brucella* isolate detected antibodies in 37% (95% CI = 27-47%) (Figure 1).

Using a McNemar's Chi square test, the correlation between the different assays showed great differences between results with the plate test having the greatest rate of positive reactions. All tests for anti-*Brucella* antibody showed significant differences ($P < 0.001$) in pair-wise comparisons, except the card test done at CSU compared to the card test at UAF (Table 1).

Discussion

This study is an extension of a previous assessment of exposure of infectious agents in this declining harbor seal population of GBNP [11], our work on brucellosis in polar bears [22] and moose (*Alces alces*) [23,24], and

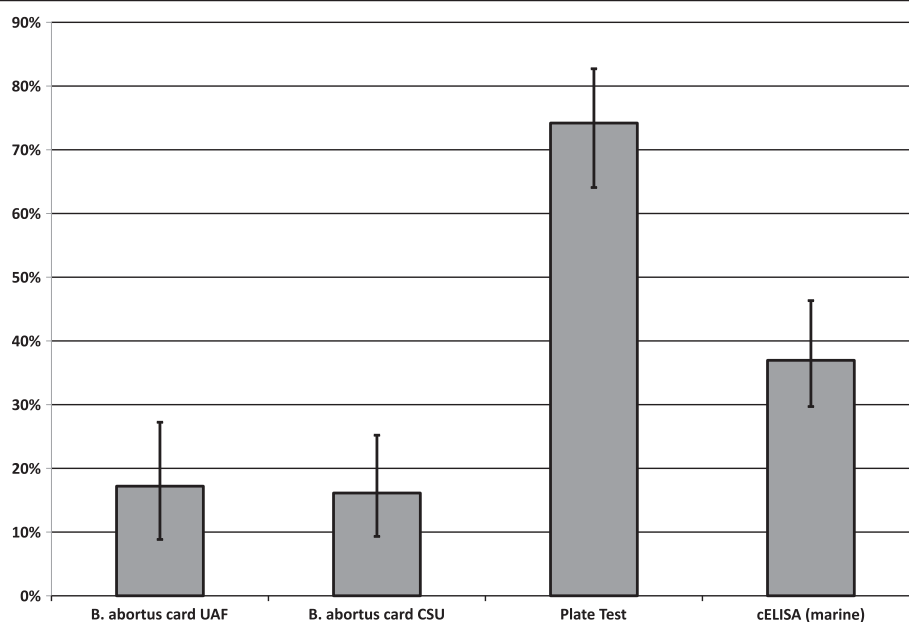


Figure 1 Serological prevalence of anti-*Brucella* antibodies for different tests. The percentage of positive reaction is shown with a 95% upper and lower confidence interval. Assays for antibodies towards *B. ovis* and *B. canis* did not detect antibodies in any samples (data not presented).

of other investigators working on Alaska mammals [12,25]. In the previous GBNP study that used the same serum samples as the present study, antibodies to *Leptospira* ssp. were detected while anti-distemper antibodies were not detected, potentially leaving the population in Glacier Bay vulnerable to this severe viral disease [11]. To assess if exposure to *Brucella* bacteria was evident and could be involved in the past and current population decline in the Glacier Bay harbor seal population, we tested serum from harbor seals from this area for the presence of anti-*Brucella* antibodies. Based on the uncertainty of *Brucella* species present in pinnipeds in the North Pacific as well as assay dependent, cross reactivity with other bacterial pathogens [26] we tested the sera using six different assays. Serological prevalence rates differed between assays detecting antibodies over a range of 16 to 74 percent of samples. Two assays failed to detect antibody, which was expected as *B. ovis* and *B. canis* have not been described in harbor seals previously and would likely not cross react with *Brucella* organism (s) known to be circulating in harbor seal populations as they have rough lipopolysaccharides (LPS), indicating that the harbor seal most likely have been exposed to *Brucella* species exhibiting a smooth phenotype. These results indicate that *Brucella* bacteria are present in the harbor seal population of Glacier Bay; however the actual serological prevalence rate cannot be determined as different assays gave widely varying results. Prevalence of anti-*Brucella* antibodies ranged from 0 to 81 percent in other studies [20,27-31] with variation attributed to annual differences [30] and assay type [29]. Together, these

findings highlight the need for cautious interpretation of serological results of exposure to *Brucella* bacteria.

Comparing the results from diagnostic tests without knowing the true prevalence of positive samples is difficult. No validated gold standard exists for the detection of antibodies against *Brucella* in pinnipeds, and developing such a standard for all marine mammal species and *Brucella* species is logistically difficult and cost prohibitive [27]. Using total predictive agreement as a measure, the plate test shows less than 55% agreement with the other tests. The card test and the cELISA agreed reasonably well, with over 70% total predictive agreement (Table 1). While this agreement is useful at a population level, it is less useful for assessing the status of individual animals. The apparent higher serological prevalence using the plate test could be explained by the conversion of fibrinogen to fibrin, which can lead to false positive results [27]; cross reactivity with antibodies to other gram negative bacteria could be an alternative explanation. However, the card test employed the same antigen and did not result in such high prevalence rates, indicating that cross reactivity of antibodies against other pathogens with the antigen used in this assay is not the main reason for the higher rate of positive samples when using the plate test.

Due to the assay dependence of serological prevalence and the uncertain pathology of brucellosis in pinnipeds, the influence of brucellosis on the harbor seal population in GBNP cannot be established. Because the serological tests used in this study are designed to detect past exposure, no conclusions can be drawn on current infection status or if *Brucella* bacteria are causing

Table 1 Pair-wise comparison of results from different serological tests

A	UAF card neg	UAF card pos	Total
cELISA neg	57	1	58
cELISA pos	19	15	34
Total	76	16	92
	NPA: 75	PPA: 93	TPA: 78
	McNemar's χ^2 : 14.45	$p < 0.001$	
B	UAF card neg	UAF card pos	Total
plate neg	24	0	24
plate pos	53	16	69
Total	77	16	93
	NPA: 31	PPA: 100	TPA: 43
	McNemar's χ^2 : 51.01	$p < 0.001$	
C	UAF card neg	UAF card pos	Total
CSU card neg	75	3	78
CSU card pos	2	13	15
Total	77	16	93
	NPA: 97	PPA: 81	TPA: 95
	McNemar's χ^2 : 0	$p > 0.1$	
D	plate neg	plate pos	Total
cELISA neg	20	38	58
cELISA pos	4	30	34
Total	24	68	92
	NPA: 83	PPA: 44	TPA: 54
	McNemar's χ^2 : 25.93	$p < 0.001$	
E	plate neg	plate pos	Total
CSU card neg	23	55	78
CSU card pos	1	14	15
Total	24	69	93
	NPA: 96	PPA: 20	TPA: 40
	McNemar's χ^2 : 50.16	$p < 0.001$	
F	cELISA neg	cELISA pos	Total
CSU card neg	55	22	77
CSU card pos	3	12	15
Total	58	34	92
	NPA: 95	PPA: 35	TPA: 73
	McNemar's χ^2 : 12.96	$p < 0.001$	

The results for two tests were compared for all samples to determine relationship between results of different tests. Test agreement is represented as negative (NPA), positive (PPA) and total (TPA) predictive agreement. Results for the McNemar's χ^2 test are represented as χ^2 and the corresponding p value for 1 df. Assays for *B. ovis* and *B. canis* results are not included here.

adverse health effects. However, a potential role of Brucellosis in the possible multifactorial population decline cannot be excluded. In addition our results show the presence of a zoonotic agent in this population, which should be taken into consideration in any management and animal handling decisions.

The assay dependent differences in apparent exposure rates to *Brucella* bacteria reinforce the need for a careful

approach to comparing literature on exposure to *Brucella* bacteria in pinnipeds using common diagnostic approaches, which are often not developed for or validated in marine mammals. While biological factors, such as age of animals sampled [12] are important in comparing different studies on *Brucella* exposure prevalence in pinnipeds, the serological test used should be considered an additional and possibly the main source of variation. Studies can only be compared and used to determine trends if similar assays were used in previous studies employing similar methods (including criteria to deem a sample positive). As most commonly used tests for antibodies against *Brucella* are based on methodology developed for livestock, companion animals, or humans, the marine cELISA used in this study is likely the most appropriate among the tests used here. In addition this test has been validated in detecting antibodies in harbor seals with brucellosis. It's also a competitive ELISA and thus increases the usefulness across different host taxa as reactivity to antibodies of the host by secondary antibodies is not necessary. However the possibility that different biovars exist between *B. pinnipedia* and uncertainty about *Brucella* spp. distribution in marine mammals should lead to careful interpretation of even this assay, which is based on a marine derived *Brucella* isolate. We strongly suggest that if results from a study are intended to be compared to previously conducted serosurveys in pinnipeds that the same test from the historic study be included alongside the marine based cELISA.

Conclusions

In this study we show that the declining harbor seal population in GBNP has been exposed to *Brucella* bacteria and that these pathogens could play a role in affecting the health of this population. Prevalence of antibodies against *Brucella* bacteria varied greatly between assays. Based on validation of this assay in harbor seals described in the literature the marine based cELISA is identified as the most appropriate test employed here.

Competing interests

The authors declare no competing interests.

Authors' contributions

TMO and SMG conceived and performed the study. KH analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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