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BRUCELLA SP. ANTIBODIES IN POLAR BEARS FROM SVALBARD AND THE BARENTS SEA

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ABSTRACT: A prevalence of 5.4% of anti-*Brucella* sp. antibodies was found in plasma samples from 297 polar bears (*Ursus maritimus*) from Svalbard and the Barents Sea. Plasma was tested by the classical brucellosis tests Slow Agglutination of Wright (SAW), EDTA modified SAW and Rose Bengal test, as well as by an indirect Protein A ELISA. Only samples classified as positive in all tests were regarded as containing anti-*Brucella* sp. antibodies. A significant west to east increase in the proportion of bears with anti-*Brucella* sp. antibodies was found, with 3.6% (n = 253) at Svalbard (Spitsbergen, Nordaustlandet, Edgeøya, Barentsøya and Hopen), and 15.9% (n = 44) in the central Barents Sea. Anti-*Brucella* sp. antibodies were previously found in ringed seals (*Phoca hispida*) and harp seals (*Phoca groenlandica*) from the same geographical areas. The ringed seal is an important prey species for the Svalbard polar bear population, and may thus be a source of brucellosis for the bears. There are no indications of reproductive disorders caused by *Brucella* sp. or other infectious agents in our study polar bear population. Potential impacts of *Brucella* sp. exposure on individuals or the population are unknown.

Key words: *Brucella* sp., brucellosis, carnivore, ELISA, marine mammal, Polar bear, serosurvey, ursus maritimus.

INTRODUCTION

Bacteria of the genus *Brucella* are small, Gram-negative, non-motile, and non-sporeforming rods that can cause highly contagious infections leading to reproductive disorders. Brucellosis has been reported throughout the world, and is an important disease in cattle, goats and swine as well as humans (Metcalf et al., 1994; Young, 1995). The disease also has been recognized in other domestic animals and a variety of wild mammals (Witter, 1981; Davis, 1990).

During the last decade, anti-*Brucella* sp. antibodies have been detected in a wide range of marine mammal species (Ross et al., 1994, 1996; Nielsen et al., 1996; Foster et al., 1996; Jepson et al., 1997; Tryland et al., 1999). Isolates of *Brucella* have been obtained from tissues from several marine mammal species (Ross et al., 1994, 1996; Foster et al., 1996; Clavareau et al., 1998). *Brucella* sp. has been isolated from an aborted bottlenose dolphin (*Tursiops truncatus*) fetus in captivity, and the bacteria were suggested as the cause of the abortion (Ewalt et al., 1994). Placentitis was diagnosed in two captive bottlenose dolphins who had aborted their 9-mo-old fetuses and *Brucella* sp. was isolated from necropsy specimens (Miller et al., 1999). Therefore, it has been concluded that brucellosis in marine mammals may be responsible for reproductive disorders and may play a role in population dynamics. Recently, exposure of a laboratory worker to a marine *Brucella* sp. isolate revealed that such bacteria also may be pathogenic to humans (Brew et al., 1999).

Characterization of marine mammal *Brucella* spp. isolates have shown that they share several features with the six defined species of the genus and thus belong to this genus, although in some respect, they constitute one or more subgroups of which new species names have been suggested (Ewalt et al., 1994; Jahans et al., 1997; Clavareau et al., 1998; Jensen et al., 1999; Bricker et al., 2000).

Anti-*Brucella* sp. antibodies have re-

cently been reported in seal and dolphin species in Antarctica and along the coast of Peru (Retamal et al., 2000; Van Bressem et al., 2001). The serological screenings conducted in the northern hemisphere indicated that *Brucella* sp. infections have a wide geographical distribution among marine mammal species (Nielsen et al., 1996; Jepson et al., 1997). We have previously reported anti-*Brucella* sp. antibodies in ringed seals (*Phoca hispida*), hooded seals (*Cystophora cristata*), harp seals (*Phoca groenlandica*), minke whales (*Balaenoptera acutorostrata*), fin whales (*Balaenoptera physalus*) and sei whales (*Balaenoptera borealis*) in the north Atlantic Ocean and the Barents Sea (Tryland et al., 1999).

The polar bear (*Ursus maritimus*) is the apex predator in the arctic marine food web, and in the Svalbard area, especially ringed seals, but also bearded seals (*Erignathus barbatus*) and harp seals are important prey species. Other species, such as walrus (*Odobenus rosmarus*), white whales (*Delphinapterus leucas*), Svalbard reindeer (*Rangifer tarandus platyrhyncus*) and various other mammals and birds, can also form part of the diet (Lønø, 1970; Smith and Lyderson, 1991; Derocher et al., 2000). In the seals previously investigated for anti-*Brucella* sp. antibodies in the Svalbard area, a seroprevalence of 10% ($n = 49$) and 2% ($n = 811$) were found for ringed and harp seals respectively (Tryland et al., 1999).

The reports of marine *Brucella* sp. isolates causing placentitis and abortions in captive dolphins highlights the possible role of *Brucella* sp. as a potential factor influencing the population dynamics of marine mammals, including polar bears. Our objective was to investigate possible exposure of polar bears at Svalbard and surrounding waters to *Brucella* sp.

MATERIALS AND METHODS

The bears were captured as a part of a research program on the Svalbard population of polar bears and plasma samples were made available for this study. The bears were captured on the sea ice in the central Barents Sea (74–77°N, 37–43°E) and on the islands and the surrounding sea ice at Spitsbergen, Nordaustlandet, Edgeøya, Barentsøya and Hopen Island, Svalbard (74–81°N, 15–30°E) from late March to mid May in 1990–1998. Yearlings and older polar bears were captured by remote injection of a drug filled dart (Palmer Cap-Chur Equipment, Douglasville, Georgia, USA) fired from a helicopter. Bears <1-yr-old were captured by hand injection of drug. The drug Zoletil vet.® (a 1:1 mixture by weight of the dissociative anesthetic Tiletamine HCl and the tranquilizer Zolazepam HCl; Virbac International, Carros Cedex, France) was administered in a solution of 200 mg/ml at a dosage of 5 to 10 mg/kg of body mass (Stirling et al., 1989). A rudimentary premolar tooth was extracted from all bears more than one-yr-old for age determination using the methods of Calvert and Ramsay (1998). The sex, reproductive status and a series of standardized morphometric measure were collected from each bear. Animal handling methods were approved by the National Animal Research Authority (NARA; Norwegian Animal Health Authority, Oslo, Norway).

Blood samples were collected from the femoral vein directly into heparinized evacuated containers. The blood samples were transferred to the laboratory and plasma was prepared and frozen within 8 hr of sampling.

All plasma samples were tested by the classical brucellosis tests Slow Agglutination of Wright (SAW), EDTA-modified Slow Agglutination of Wright (SAW-EDTA) and Rose Bengal Test (RB). The tests were performed according to Alton et al. (1988). The tests were interpreted as when used for testing cattle. For the SAW and SAW-EDTA, samples with titers ≥30 IU were considered positive. For the RB test, any degree of agglutination (scored 1+ to 4+) was considered positive. Since the samples tested are plasma rather than serum, the classical brucellosis Complement Fixation Test (CFT) could not be used. Presence of *Brucella*-specific immunoglobulin G (IgG) was therefore investigated by an ancillary test, an indirect ELISA. To use the indirect Protein-G ELISA designed for detecting anti-*Brucella* sp. antibodies in cattle (Limet et al., 1988) and previously used for several marine mammal species together with the classical brucellosis tests (Tryland et al., 1999), we checked the affinity between polar bear immunoglobulin molecules to Protein-G and Protein-A. Microtiter plates were coated with polar bear plasma and cattle serum and incubated with biotinylated Protein-G and Protein-A. Streptavidin peroxidase were added followed by orthophenylenediamine as substrate. Due to an apparent lack of affinity

TABLE 1. A comparison of the test results from the three classical brucellosis tests (SAW, SAW-EDTA and RB) and the Protein-A ELISA when testing 297 polar bear plasma samples. Samples defined positive by each test are in bold.

Titer (SAW & SAW-EDTA)	SAW	SAW-EDTA	RB (aggl. score)	Protein-A ELISA
			276 (0)	
≤25	279	280	**1** (1+)	
30	**1**	**1**	**5** (2+)	
50	**9**	**7**	**2** (3+)	
100	**8**	**9**	**13** (4+)	
Positive samples (%)	**18** (6.0%)	**16** (5.4%)	**21** (7.0%)	**157** (53%)

between polar bear immunoglobulins and Protein-G (data not shown), the ELISA was performed as described previously (Tryland et al., 1999) with *Brucella* lipopolysaccharide (LPS) as antigen, except that a biotinylated Protein-A conjugate (Sigma-Aldrich Norway AS, Oslo, Norway) was used instead of Protein-G. The Protein-A conjugate was diluted 1:300 in phosphate buffered saline containing 0.1% Tween 20 (PBS-T) and incubated at room temperature for 1 hr, followed by 50 μl streptavidin-peroxidase (Streptavidin-POD conjugate; Boehringer Mannheim, GmbH, Mannheim, Germany), 1000 U/ml diluted 1:1,000 in PBS-T, that was incubated at room temperature in the dark for 30 min. Polar bear samples with a verified presence or absence of anti-*Brucella* sp. antibodies (positive and negative control sera) were not available. A bovine *B. abortus* hyperimmune standard serum with 1,200 EU CFT units was used as positive control in dilutions from 1:1,000 to 1:32,000 (corresponding to 60 to 1.875 units). Due to possible species differences concerning affinity between immunoglobulins and Protein-A, the results from the cattle control serum could not be directly compared with the measurements of polar bear samples. As an interpretation of the ELISA, a cut-off optical density (OD) value was calculated as the mean OD of the 100 plasma samples with lowest OD value + three standard deviations (Duncan, 1988).

In order to detect non anti-LPS antibodies present in these samples, an ELISA based on *Brucella* cytoplasmic proteins was used. The Brucellergene OCB (Symbiotics Corporation, Lyon, France), which consists mainly of cytoplasmic proteins and which is used as allergen in the brucellosis skin test (Saegerman et al., 1999), was used as antigen in an ELISA. Microtiter plates were coated with 100 μl of Brucellergene OCB (batch 86W221; 0.5 mg/ml) and blocked with 3% bovine serum albumin (BSA) in PBS. Polar bear samples were diluted 1/50 and the assay was performed as described previously (Limet et al., 1988), except that a Protein A-HRPO conjugate (Nycomed Amersham, Buckinghamshire, UK) was used instead of a Protein-G conjugate. Nine of the 16 individuals classified as having anti-*Brucella* sp. antibodies and 10 individuals classified as negative were tested with the Brucellergene ELISA.

Agreement between the different assays were tested by calculating kappa (κ) (Martin et al., 1987). Yates corrected chi-squared test (Altman, 1991) and the student *t*-test were used to search for associations between the presence of anti-*Brucella* sp. antibodies and other parameters (Statistix® 4.1 software package; Analytical Software Co., Tallahassee, Florida, USA). Statistical significance in this paper refers to the 5% level.

RESULTS

The 297 polar bears (150 males and 147 females) were captured in 1990 (n = 1), 1991 (n = 5), 1992 (n = 3), 1993 (n = 9), 1994 (n = 7), 1995 (n = 23), 1996 (n = 23), 1997 (n = 36), and 1998 (n = 190). The age of the bears ranged from 3 mo to 28 yr ($\bar{x}$ = 9 yr). Of the 147 females, 87 were ≥5-yr-old and were considered to be sexually mature.

We tested the 297 polar bear plasma samples for anti-*Brucella* sp. antibodies (Table 1). To achieve the highest degree of specificity, only the sixteen samples (5.4%) that were defined positive in all the classical brucellosis tests, and had an OD above the calculated cut-off value in the indirect Protein-A ELISA were regarded as true positives. There was a high correlation between the SAW and the SAW-EDTA tests when comparing titers (κ = 0.99). Comparing positive reacting plasma

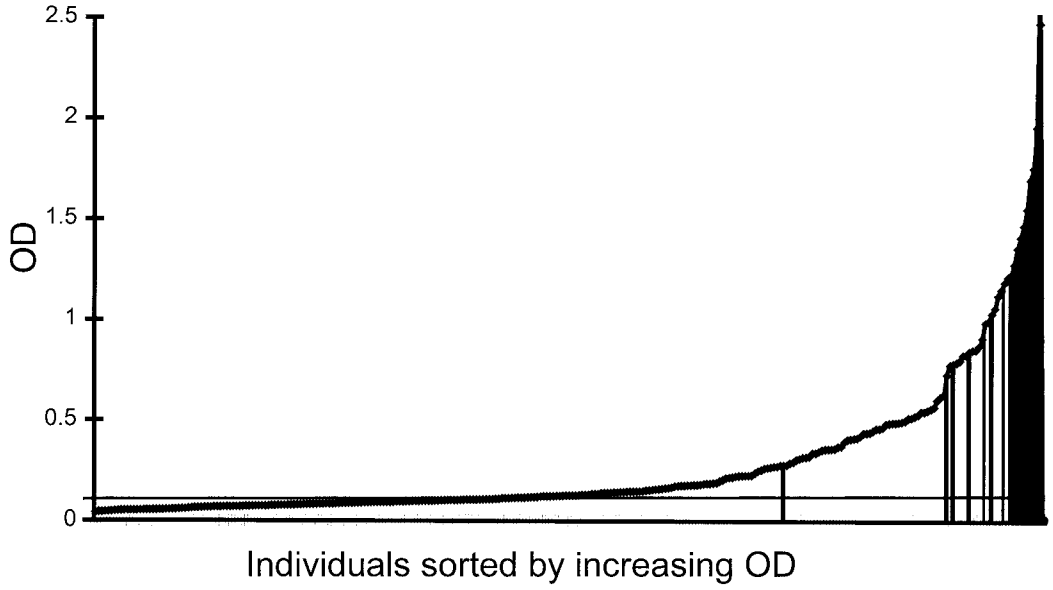

FIGURE 1. Distribution of optical density (OD) values for polar bear plasma in the Protein-A ELISA, compared with the results from the classical brucellosis test (SAW, SAW-EDTA and RB). Each black bar represents a plasma sample defined positive in all the three classical brucellosis tests, all of which had OD values above the cut-off level for the ELISA (horizontal line).

samples in the SAW (titer ≥30) and the RB test (≥1+) also revealed good agreement ($\kappa = 0.75$). A comparison of the samples defined positive by all the three classical tests and the distribution of OD values obtained by the Protein-A ELISA were grouped (Fig. 1). The indirect Protein-A ELISA revealed OD values ranging from 0.043 to 2.47, whereas blank wells (buffer controls) had an OD range from 0.039 to 0.066 (mean 0.049, standard deviation 0.0079). For the 100 samples with the lowest OD value, the OD ranged from 0.043 to 0.096 (mean 0.073, standard deviation 0.015). Using the calculated cut-off for the ELISA (OD = 0.118), 157 of the 297 individuals (53%) should be regarded as having anti-*Brucella* sp. antibodies by this test alone.

Nine of the 16 individuals classified as having anti-*Brucella* sp. antibodies by the SAW, SAW-EDTA, the RB and the indirect Protein A-ELISA, had a mean OD value in the Brucellergene ELISA of 1.284, which were significantly higher ($P < 0.001$) than the mean OD value when testing 10 individuals classified as not having anti-*Brucella* sp. antibodies by the SAW, SAW-EDTA, the RB and the indirect Protein-A ELISA (0.572).

Of the 16 individuals with anti-*Brucella* sp. antibodies, nine were males and seven females. Bears with anti-*Brucella* sp. antibodies ranged from 1 to 25 yr old ($\bar{x} = 10$ yr), and where found from the northern part of Svalbard (Ekstremhuken, Nordaustlandet) to the central Barents Sea. The prevalence of anti-*Brucella* sp. antibodies among the bears sampled in the central Barents Sea was 15.9% ($n = 44$), which was three times as high as the mean prevalence, and significantly higher than among bears sampled at Svalbard (including Hopen Island), where a prevalence of 3.6% ($n = 253$) was found. The prevalence among polar bears sampled at Hopen Island was 5.4% ($n = 130$), which was significantly lower than found in the Central

Barents Sea, but not significantly higher than for the rest of Svalbard (Spitsbergen, Nordaustlandet, Barentsøya and Edgeøya), where a prevalence of 1.6% (n = 123) was found.

There were no significant differences in sex or age composition among bears sampled on the different locations. Most of the samples, with and without anti-*Brucella* sp. antibodies, were obtained in 1998, and no significant correlation was found between the presence of antibodies and sampling year. A significant difference in prevalence was found when comparing samples from the central Barents Sea (15.9%, n = 44, all from 1998) with the other samples from 1998 (4%, n = 156).

Of the 87 sexually mature females, seven carried anti-*Brucella* sp. antibodies. Three of these animals (7-, 12-, and 15-yr-old) had cubs-of-the-year, while the rest had no cubs. Of the remaining 80 mature females classified negative with regard to anti-*Brucella* sp. antibodies, 45 individuals (56%) had cubs (0-2 yr-old). A total of 40 cubs-of-the-year were tested, including the three with anti-*Brucella* antibodies positive mothers, and all were classified as negative. The youngest individual classified as positive was a one-yr-old male, whose mother, a 14-yr-old female, was classified as negative.

Due to an apparently lack of affinity between polar bear immunoglobulins and Protein-G, Protein-A should be chosen, when anti-polar bear antibodies are not available.

DISCUSSION

There are no previous reports on the presence of anti-*Brucella* antibodies or isolation of bacteria belonging to the genus *Brucella* from polar bears. The variety of brucellosis indirect test results are, in general, best supported by the isolation of *Brucella* sp. from individuals in the animal population tested. However, samples other than blood were unavailable for this study. In non-infected cattle, a high proportion of the *Brucella* agglutinating activity present in the serum has been shown to be EDTA labile (MacMillan and Cockrem, 1985). In our study, we did not find EDTA labile agglutinins in the polar bear plasma, suggesting that the agglutination observed was specific or due to cross-reacting antibodies, possibly against *Yersinia enterocolitica* O:9, as reported to occur in cattle (Weynants et al., 1996). In a previous study, cross-reacting antibodies against *Yersinia enterocolitica* 0:9 were not found in sera from seals and whales, and we were not able to cultivate *Yersinia enterocolitica* from various tissues from 62 individuals (Tryland et al., 1999). Further, there are no reports on isolation of *Y. enterocolitica* O:9 from seals or other marine mammals. In the polar bear plasma samples, some anti-*Yersinia enterocolitica* O:9 agglutination could be seen in some of the samples that were classified as having anti-*Brucella* sp. antibodies by the classical tests and the Protein-A ELISA, although with low titers that did not suggest an infection with *Y. enterocolitica* O:9 in these animals (data not shown). The subsequent detection of specific antibodies directed against *Brucella* cytoplasmic proteins by the Brucellergene ELISA, in addition to the previous mentioned results from the classical brucellosis tests and the LPS-based Protein-A ELISA, strongly suggests that the antibodies detected in this study are due to a *Brucella* sp. infection.

In cattle, the Protein-G brucellosis ELISA is a quantitative assay and a value of 1.875 units is used as the standard cut-off. Since Protein-G had very weak affinity to immunoglobulins from polar bears, we changed to Protein-A. In cattle, Protein-A is reported to bind a smaller fraction of the immunoglobulins compared to Protein-G (Richman et al., 1982), whereas no such information exists for polar bears. Since we did not have positive brucellosis control serum or plasma from polar bears, or verified negative serum samples from this species, it was not possible to calculate the specificity and sensitivity for the test, or to compare the OD values directly with val-

ues from the bovine positive control serum. We have therefore chosen to use a calculated cut-off value for the Protein-A ELISA based on a group of samples with a low OD value in the test, and which also tested negative in the three classical brucellosis tests, i.e., samples where anti-*Brucella* sp. antibodies most likely are absent.

To strengthen the specificity of the detection of anti-*Brucella* sp. antibodies, we chose a parallel interpretation of the tests used. Using these criteria, the prevalence of anti-*Brucella* sp. antibodies may be underestimated and the prevalence of 5.4% may be regarded as a minimum figure. This was supported by the high number of individuals above the cut-off level in the indirect Protein-A ELISA that were classified as negative because they were negative in one or more of the classical tests. For bovine sera, the classical brucellosis tests had a lower sensitivity than a comparable indirect ELISA. The discrepancy between the indirect Protein-A ELISA and the classical brucellosis tests may thus partly suggest a higher sensitivity in the ELISA, although we can not rule out a lack of specificity. However, in one recent study infected cattle were classified negative by agglutination tests and positive by an indirect ELISA test in the case of chronic infections (Saegerman et al., 1999). In the Belgian population of wild boars (*Sus scrofa*), the SAW was found to be unsatisfactory for the detection of an unrecognized enzootic brucellosis, whereas the Protein-G ELISA was able to detect infected animals on all the hunting grounds where *Brucella suis* biovar 2 was isolated from infected animals (Godfroid et al., 1994). All together, these data suggest that the antibodies detected in the polar bear samples may have been induced by a *Brucella* sp. infection.

The Protein-A ELISA may be a useful and sensitive tool for detecting anti-*Brucella* sp. antibodies in polar bears. However, to be able to calculate a precise cut-off OD value for future studies, and to determine whether brucellosis exists as chronic infections in the polar bear population, bacteriological work must be conducted. As compared to the isolation method of *Brucella suis* biovar 2 performed on wild boars, tonsil swabs may be the best method for the isolation of *Brucella* sp. from wild animal species (Godfroid et al., 1994).

The higher prevalence of anti-*Brucella* sp. antibodies among polar bears from the central Barents Sea compared to those from Svalbard is of interest. The trend could not be explained by different sex or age composition or sampling year. Satellite tracking and mark-recapture data previously suggested the Svalbard population of polar bears to be separated from the Franz Josef Land/Novaya Zemlya and the east Greenland populations (Wiig, 1995). However, recent telemetry studies and genetic mapping indicated that cross-border movements are common, especially between the Svalbard and the Franz Josef Land/Novaya Zemlya populations (A. E. Derocher and Ø. Wiig, unpubl. data), and that these two populations are closely linked genetically (Paetkau et al., 1999). The home range sizes for the Svalbard population of polar bears varies from 1,200 km^2 to 250,000 km^2 and, generally, polar bears in the central Barents Sea region have the largest home ranges and move further east than the polar bears marked on the east coast of Spitsbergen (Wiig, 1995). The central Barents Sea individuals may have greater access to harp seals compared to bears closer to Svalbard. Thus, the geographic variation in anti-*Brucella* sp. antibodies among the polar bears may reflect the prevalence of infection in prey. However, ringed seals tested for anti-*Brucella* sp. antibodies had a seroprevalence of 4% ($n = 27$) between Novaya Zemlya and Franz Josef Land and 40% ($n = 10$) from the coast of Spitsbergen (Tryland et al., 1999). Although the number of seals tested was limited, the data is opposite to the pattern in prevalence of such antibodies of polar bears from the different regions. For harp seals, the seroprevalence

was 1% (n = 299) near Kapp Kanin («East Ice») and 5% (n = 21) between Novaya Zemlya and Franz Josef Land (Tryland et al., 1999). Seals in both areas belong to the Barents Sea stock of harp seals that breed and molt in the White Sea and surrounding waters (Haug et al., 1994). Therefore, the prevalence of anti-*Brucella* sp. antibodies can differ between regions within the same population. Considering the wide distribution of anti-*Brucella* sp. antibodies in different sea mammal species it is reasonable that such antibodies, and the bacteria, are generally present in the different seal populations in the Barents Sea. Thus, we believe that ingestion of infected seal tissue is a likely source of infection for the polar bear. However, other infection sources are possible, such as individual to individual transmission by ingestion of infected material from birth, as is a common source of other *Brucella* spp. among other terrestrial mammals.

The presence and frequency of diseases in this polar bear population are largely unknown. The population appears healthy and no serious disease problem has been registered, although specific antibodies against canine distemper virus and calicivirus have been identified in some individuals (M. Tryland et al., unpubl. data). However, this population of polar bears is heavily exposed to organochlorines (Bernhoft et al., 1997; Norstrom et al., 1998) which may influence immune response and alter the levels of IgG (Tizard, 1996). For this population of polar bears, a significant decrease of blood IgG levels was found with increasing polychlorinated biphenyls (PCB) and hexachlorobenzene (HCB) concentrations. Such pollutants may have impaired immune function and made the polar bears more susceptible to infectious agents and diseases (Bernhoft et al., 2000).

Under normal circumstances, polar bears have a three-year breeding cycle and breed after the cubs are weaned at two yrs of age (Ramsay and Stirling, 1988; Wiig 1998). In our data, the proportion of sexually mature females known to have reproduced (i.e., that were followed by cubs-of-the-year, yearlings or 2-yr-old cubs) was 55%. This proportion appears normal when mortality of young cubs is considered. The data are too restricted to draw any conclusions about *Brucella* related reproductive disorders among female polar bears. Of the seven mature females with anti-*Brucella* sp. antibodies, 43% had cubs (3/7), while 56% (45/80) of mature females classified as negative had cubs.

Thus, although bacteria belonging to the genus *Brucella* have caused placentitis and abortion in captive bottlenose dolphins, and that anti-*Brucella* sp. antibodies have been detected in the Svalbard population of polar bears, the impact of *Brucella* sp. infections in these animals is unknown. Further investigations are needed, including bacteriological work, on the role of *Brucella* infections in the Svalbard polar bear population.

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