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Influenza virus infection in seal (*Phocidae*): seroepidemiological survey of influenza virus in Caspian seals (*Phoca caspica*)

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In the last a few decades, several viral diseases in marine mammals such as seals and cetaceans were characterized. Influenza virus causes a worldwide zoonosis, influenza, and was shown to be involved in mass mortality in seals. Several influenza virus strains have been isolated from the sick seals. Because interspecies transmission of influenza virus plays a crucial role in the introduction of pandemic influenza disease in humans, it is important to monitor the virus distribution in wild animals including marine mammals. In this article, we review the previous findings on influenza virus infection in seals, and introduce our recent serological surveillance of influenza virus in Caspian seals (*Phoca caspica*) in 1993–2000. Our recent results suggested that the Caspian seals were infected by human related influenza viruses. The possibility of seals as reservoirs of influenza virus, and the importance of surveillance of the virus infection in marine mammals have been discussed.

Key words: influenza virus, seal, marine mammals

INTRODUCTION

Recent outbreaks of mass mortality and stranding in marine mammals such as seals and cetaceans are a serious problem not only for the protection of the wild animals but also for public health. Infectious disease is thought to be one of the major causes of these events. Influenza virus belonging to *Orthomyxoviridae* was shown to be involved in mass mortality in seals. Influenza virus is divided into three types, A, B, and C. Influenza A virus infects a variety of avian and mammalian species including humans (Webster et al. 1992). Mass-die-off of harbor seals (*Phoca vitulina*) associated with influenza virus infection occurred on the northeast coast of the U.S.A. in 1979–1980 (Geraci et al. 1982). On the other hand, whilst influenza B and C viruses had been believed to be a pathogen only for humans, the infection of seals with influenza B virus has been reported recently (Osterhaus et al. 2000). Interspecies transmission of the influenza virus is an important event in the evolution and ecology of the virus, and could introduce a pandemic disease to humans. It is, therefore, important to monitor the distribution of the influenza virus in wild animals. However, limited information is available in marine mammals. In this paper, we summarize previous findings of influenza virus infection of seals (*Phocidae*), and introduce our recent serological study against influenza A and B viruses in Caspian seals (*Phoca caspica*) in 1993–2000 (Ohishi et al. 2002).

PREVIOUS REPORTS OF INFLUENZA VIRUS INFECTION IN SEALS

(1) Influenza A virus infection in seals

Mass-die-off of harbor seals (*Phoca vitulina*) associated with a severe pneumonia, occurred in Cape Cod, Massachusetts, U.S.A. in December 1979 to October 1980 (Geraci et al. 1982). Approximately 600 animals were estimated to be dead during this outbreak of the disease. Ninety percent of the dead seals were less than 3 years old (Geraci et al. 1982). Pneumonia characterized by necrotizing bronchitis and bronchiolitis, and hemorrhagic alveolitis, were found on postmortem examination (Geraci et al. 1982). H7N7 influenza A virus (A/seal/Massachusetts/1/80) was isolated from the lungs and brains of the dead animals (Webster et al. 1981b, Lang et al. 1981). Genetic and antigenic studies showed the seal virus originated from birds (Webster et al. 1981b, Lang et al. 1981, Kida et al. 1982). The H7N7 virus could have infected humans and squirrel monkeys, and could have caused conjunctivitis or pneumonia (Murphy et al. 1983, Webster et al. 1981a). The virus replicated in the respiratory tract of pigs, cats, and ferrets. In contrast, the virus did not replicate in the cells lining the intestinal tract of ducks, and not be isolated from fecal samples of birds (Webster et al. 1981b). These results

indicated that the seal virus behaved biologically more like a mammalian strain than like an avian strain, despite the genome as a whole seemed to originate from birds.

Some other subtypes of influenza A viruses, H4N5 virus (A/seal/Massachusetts/133/82), H4N6 virus (A/seal/Massachusetts/3807/91 and A/seal/Massachusetts/3810/91), and H3N3 virus (A/seal/Massachusetts/3911/92 and A/seal/Massachusetts/3984/92) were isolated from the lungs of dead seals in the following epizootics of pneumonia in the same place in 1982–1983 and 1991–1992 (Hinshaw et al. 1984, Callan et al. 1995). The pathogenecity of these viruses in seals seemed to be milder than that of H7N7 virus. The pathologic lesions of these lung tissues were consistent with viral pneumonia and included an acute interstitial pneumonia and subcutaneous emphysema (Hinshaw et al. 1984, Callan et al. 1995). These seal viruses were shown to originate from birds by genetic analysis (Hinshaw et al. 1984, Callan et al. 1995). H4N5 virus replicated well in intestinal tracks of the experimentally inoculated ducks (Hinshaw et al. 1984). This demonstrated that the virus had a tropism of avian strain

Recently serologic evidence of influenza A virus infection was shown using huge number of serum samples from marine mammals inhabiting arctic Canada (Nielsen et al. 2001). It reported that 2.5% (23/903) of serum samples from ringed seal (*Phoca hispida*) in arctic Canada were positive for influenza A virus antibody, although serological subtype of the virus was not determined.

(2) Influenza B virus infection in seals

Influenza B virus had been known to infect only humans until the virus was isolated from a harbor seal (Osterhaus et al. 2000). Stranded seals were found on the Dutch coast, and 12 juvenile with respiratory problems were examined in the spring of 1999. Antibodies against influenza B virus were detected in two seals, and influenza B virus was isolated from a throat swab sample of one seal (B/seal/Netherland/1/99). Sequence analyses and serological examination indicated that the virus was closely related to a strain that circulated in humans 4 to 5 years earlier. Retrospective examination of the virus-specific antibodies was conducted using the sera obtained from 580 seals before 1995 and from 391 seals during or after 1995. None of seal sera before 1995 were positive, whereas 8 seal sera during and after 1995 showed positive. Among 8 seropositive seals, six animals were harbor seal pups, and two were gray seal pups. Harbor and gray seals are known to share the same habitat in Dutch coastal waters.

These findings indicated that seals could be infected by influenza A and B viruses. However, despite the virus isolation from seals, clear serological evidence of infection with influenza viruses in seals is few.

SEROLOGICAL STUDY OF INFLUENZA VIRUS INFECTION IN CASPIAN SEALS

To know the distribution of influenza virus infection in marine mammals, serological survey that can detect the past infection is effective. The approach associated with serological and biological study including age determination will provide a precious ecological information such as the time of invasion and maintenance of the virus in the population. We constructed a monitoring system of antibodies against the virus, and conducted a serological study of Caspian seals in 1993–2000 (Ohishi et al. 2002).

(1) Samplings and biological data

Samples were obtained under the Russian-Japanese Joint Research Program for Biological and Environmental Studies of Caspian seals, on the Pearl Island (45°03′N, 48°18′E) located in the northwest area of the Caspian Sea, in November 5–11, 1993; August 15–16, 1997; September 12–16, 1998, and September 24–October 8, 2000. Thirteen (9 females, 4 males), seven (3 females, 4 males), fifteen (12 females, 3 males) and forty-two (27 females, 15 males) seals were examined in 1993, 1997, 1998 and 2000, respectively, with a special permission of the local government. After measurement of body size, serum samples were obtained from these 77 seals. Longitudinal, decalcified and haematoxylin-stained thin sections of canine teeth were prepared following a previously described procedure (Kasuya 1976). Age of Caspian seals was estimated from the higher number of either dentinal or cemental growth layers in a canine tooth. One growth layer group was assumed to correspond to 1 year. Since pup Caspian seals are born between the middle of January and the end of February and all samples were collected between late summer and autumn, age of each seal was expressed as an integer plus 0.5.

(2) Detection of antibodies to influenza A virus by enzyme-linked immunosorbent assay (ELISA) and Western blot analysis

Seal serum samples collected in 2000 were screened by ELISA (Ohishi et al. 2002). Antibodies to influenza A virus were detected in 26% (11/42) of the serum samples (Fig. 1). The only animals that were more than 14.5 years old showed positive. Although the ages of 4 seals (2 positives and 2 negatives in ELISA) could not be determined, these seals were all judged to be adults from their body length (114–124.5 cm). Antibodies to influenza A virus assessed by ELISA were detected in 54% (7/13), 57% (4/7) and 40% (6/15) of the serum samples in 1993, 1997, and 1998, respectively (Fig. 2). The ages of two seals caught in 1993 could not be determined; these seals were antibody-negative in the ELISA. Antibodies were detected only in the sera of animals older than 11.5 and 20.5 years old in 1997 and 1998, respectively, whereas in 1993, antibodies were detected in the sera from 3 pups that were younger than one year old.

The reactivity of the antibodies of the seal serum samples to influenza A viral antigens was examined by Western blot analysis according to standard procedure (Towbin et al. 1979). Fig. 3 demonstrated the typical band patterns by use of ELISA-positive seal sera. Under the reduced condition, two protein bands of approximately 50 kDa and 28 kDa corresponding to two hemagglutinin (HA) subunit protein, HA1 and HA2, were detected in the positive seal sera (Fig. 3a). Under non-reduced condition, a major band was observed around 75 kDa, corresponding to the HA protein consisting of the two subunits (HA1, HA2) linked by disulphide bonds (Fig. 3b). Because influenza A virus-immu-

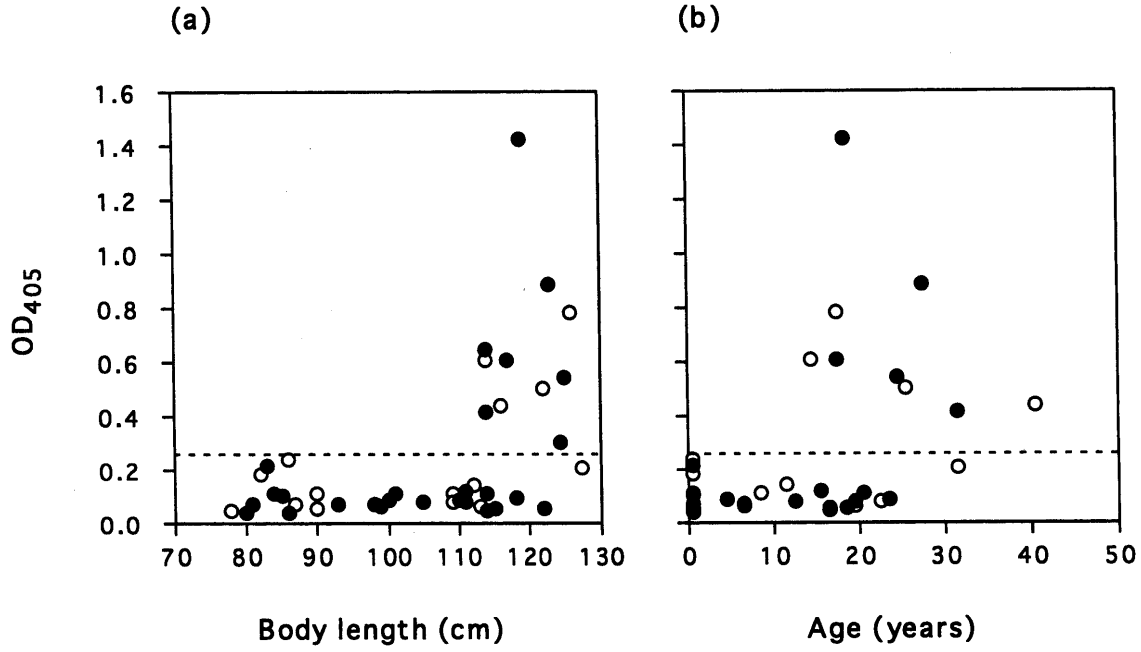

Fig. 1. Antibodies to influenza A virus by ELISA in seal sera collected in the year 2000.
Absorbance at 405 nm versus (a) their body length (cm), n=42, and (b) ages (years), n=38, is shown. Purified and dissolved A/Aichi/2/68 (H3N2) was used as antigen. The seal sera diluted to 1 : 50 and peroxidase-conjugated Protein A was used for the detection of antibodies. An absorbance value higher than 0.25, twice of the highest absorbance value of the sera from captive seals that were thought to be negative, was regarded as positive. Filled circles: females, open circles: males.

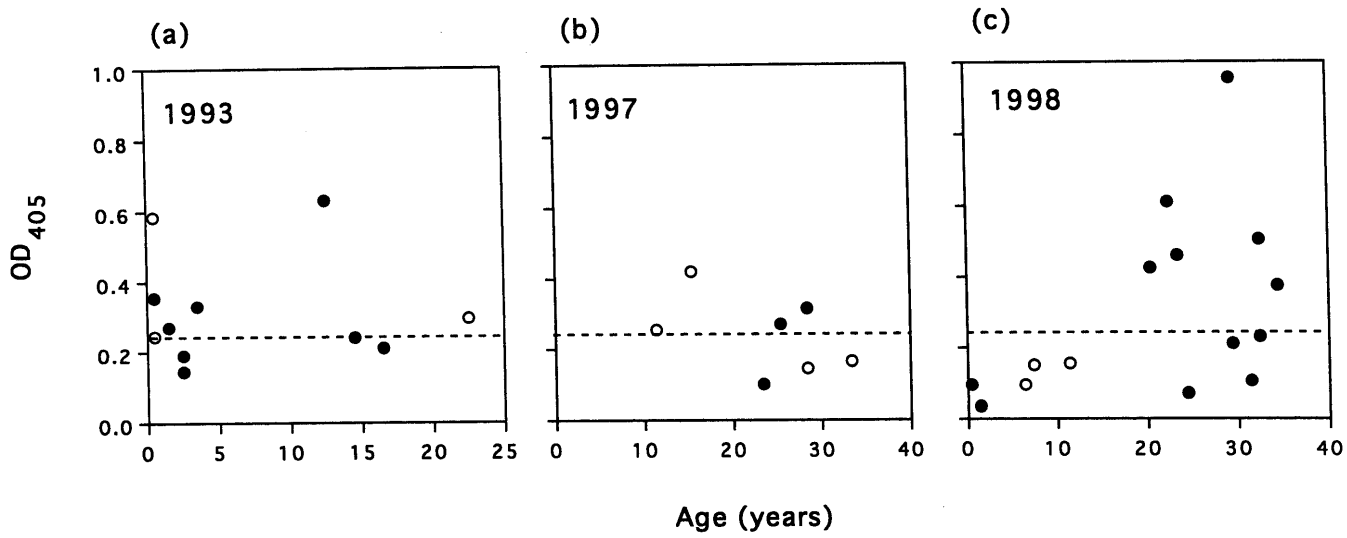

Fig. 2. Antibodies to influenza A virus by ELISA in seal sera collected in 1993–1998.
Absorbance at 405 nm versus their ages (years) is shown. Sera were collected in (a) 1993: n=15, (b) 1997: n=7, and (c) 1998: n=13. See the legends for Fig. 1.

nized mouse serum as a positive control showed the same band patterns, the Western blot analysis system was thought to detect antibodies to influenza A virus, specifically to HA of the virus. These results indicate that Caspian seals were infected by influenza A virus and that the serum antibodies of seals reacted with HA of the virus.

(3) Serological determination of subtype of influenza A virus

To determine the subtype of the HA recognized by the serum antibodies, hemagglutination-inhibition (HI) test using the reference influenza A virus strains of each of the known HA subtypes (H1–H15) was carried out (Ohishi et al. 2002). Eleven ELISA-positive sera of the 1998 and 2000 samples were used for this test because the amounts of other seal sera were very limited. Six samples inhibited hemagglutination of the A/Aichi/2/68 (H3N2) virus (Table 1(a)). To investigate the strain specificity of the antibodies detected by the above tests, the sera were further examined by HI test for their reactivity with human H3N2 naturally occurring antigenic variants, and H3 viruses of duck, swine, and equine origin. The sera strongly reacted with the A/Bangkok/1/79 (H3N2) strain, and slightly with A/Aichi/2/68 (H3N2) strain (Table 1(b)). The remaining four ELISA-positive sera collected in 2000 and one ELISA-positive serum in 1993 from a 0.5 year old pup,

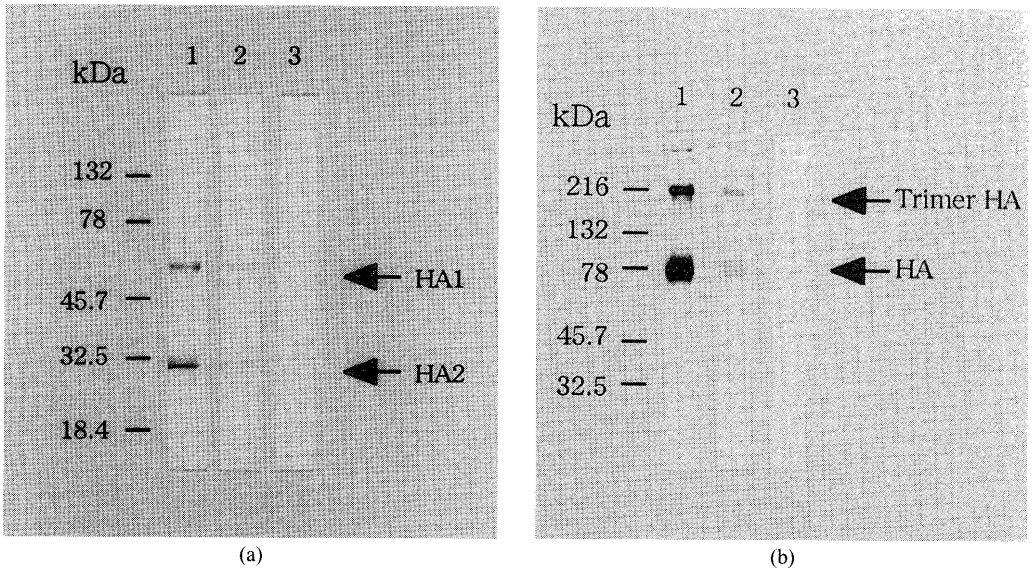

Fig. 3. Western blot analysis of the antibodies against influenza A virus in the seal sera.
A/Aichi/2/68 (H3N2) viruses (16 μg/lane) resolved under reduced (a) or non-reduced (b) conditions, were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) on 4–20% gradient polyacrylamide gels. Seal serum samples diluted at 1 : 50 and peroxidase-conjugated Protein A were used for detection of the antibodies. Viral proteins were reacted with the mouse serum immunized with A/Aichi/2/68 virus, as a positive control (lane 1); positive seal serum by the ELISA (lane 2: No.13, OD=0.891); negative seal serum (lane 3, No.14, OD=0.081).

were tested only with A/Aichi/2/68 and A/Bangkok/1/79 strains due to the small amounts. All these five sera inhibited hemagglutination of A/Bangkok/1/79 strain at high titers (titer: 512–2048) (Table 1 (b)). These results indicate that the seals caught during 1993–2000 were infected with A/Bangkok/1/79-like influenza virus.

(4) Detection of anti-influenza B virus antibodies in seal sera

ELISA using B/Lee/40 as antigens was conducted. Antibodies were detected in four among 42 seal serum samples collected in 2000 (Fig. 4). Three among four positive animals were younger than one year old, showing that the influenza B virus had recently been introduced. No influenza B virus-specific antibodies were detected in the seal sera collected in other years except for one serum sample in 1997 which showed weakly positive. In Western blot analysis, a band of around 82 kDa, corresponding to HA, was detected in positive seal serum samples as well as in chicken positive control serum under non-reduced condition (data not shown). No significant band to influenza B virus proteins was detected under reduced condition

DISCUSSION

In the last a few decades, several viral diseases in marine mammals were characterized (Kennedy-Stoskopf 2001). A series of isolations of influenza A virus from dead seals at Cape Cod stimulated the study of the infection by influenza virus on marine mammals (Webster et al. 1981b, Hinshaw et al. 1984, Callan et al. 1995). To reveal the distribution of influenza virus in marine mammals, we conducted a serological surveillance in Caspian seals. Our results demonstrated serological evidence for infection of influenza A virus in seal sera collected in 1993–2000 (Fig. 1, 2) (Ohishi et al. 2002). The sera strongly reacted with A/Bangkok/1/79 (H3N2) strain, suggesting that the seals were infected with influenza A virus that circulated among humans in 1979–1981. Although it remains unclear when the virus invaded the seal population, it is reasonable to assume that the virus invaded the Caspian seal population in the early 1980s when A/Bangkok/1/79-like viruses were also prevalent in humans. A serum sample from a pup seal caught in 1993 was positive for the virus strain, implying that A/Bangkok/1/79-like viruses were maintained in the Caspian seal population at least until 1993. The antigenic and genetic evolution of influenza A virus is so rapid in humans, in contrast in ducks and pigs (Kida et al. 1987, 1988, Webster et al. 1992). Our data indicate that the virus may have been maintained in seals in a conservative manner as in ducks and pigs after the counterpart virus has disappeared in humans. Although previous findings have shown transmission of the virus from birds to seals (Webster et al. 1981b, Hinshaw et al. 1984, Callan et al. 1995), the A/Bangkok/1/79 virus strain has never been isolated from birds. This suggests that the influenza A virus has been directly transmitted from humans to seals. On the other hand, antibodies to influenza B virus were detected in the sera from four Caspian seals including three pups in 2000 as shown Fig. 4. The finding suggests that influenza B virus infection in Caspian seals occurred recently, probably from humans. Thus, our results suggest that influenza A and B viruses were transmitted from humans to seals.

The previous and our recent findings raised the possibility that seals are reservoir of influenza viruses originated from birds and humans (Webster et al. 1981b, Hinshaw et al. 1984, Callan et al. 1995, Ohishi et al. 2002). This furthermore suggests that seals may play a role as "mixing vessels", similar to the role played by pigs, which could produce a pandemic virus strain by genetic reassortment of the viruses (Kida et al. 1994).

Surveillance of influenza virus infection in wild animals is important not only for understanding the ecology of the

Table 1. Hemagglutination-inhibition (HI) test of seal serum samples with influenza A viruses.

a

Influenza A virus strain (subtype)	Positive rate*	Range of HI titer#
A/swine/Iowa/15/30 (H1N1)	0/11	—
A/Singapore/1/57 (H2N2)	3/11	64
A/Aichi/2/68 (H3N2)	6/11	64–256
A/duck/Czechoslovakia/56 (H4N6)	0/11	—
A/duck/Pennsylvania/10128/84 (H5N2)	0/11	—
A/shearwater/Australia/1/72 (H6N5)	0/11	—
A/seal/Massachusetts/1/80 (H7N7)	0/11	—
A/turkey/Ontario/6118/67 (H8N4)	0/11	—
A/chicken/Hong Kong/G24/98 (H9N2)	0/11	—
A/chicken/Germany/N/49 (H10N7)	0/11	—
A/duck/England/1/56 (H11N6)	0/11	—
A/duck/Alberta/60/76 (H12N5)	0/11	—
A/gull/Maryland/704/77 (H13N6)	0/11	—
A/mallard/Astrakhan/263/82 (H14N5)	0/11	—
A/duck/Australia/341/83 (H15N8)	0/11	—

b

Virus strain bearing H3 HA (subtype)	Positive rate*	Range of HI titer#
A/Aichi/2/68 (H3N2)	10/16	64–256
A/Bangkok/1/79 (H3N2)	12/16	128–2048
A/Philippines/2/82 (H3N2)	0/11	—
A/Memphis/1/96 (H3N2)	0/11	—
A/sw/Hong Kong/126/82 (H3N2)	1/11	64
A/duck/Hokkaido/5/77 (H3N2)	1/11	64
A/duck/Hokkaido/33/80 (H3N8)	1/11	64
A/equine/Miami/1/63 (H3N8)	0/11	—
A/equine/Kentucky/1/81 (H3N8)	0/11	—
A/equine/La Plata/1/93 (H3N8)	0/11	—

HI titer was given as the highest serum dilution that inhibited 4 units of hemagglutination. The titer higher than 64 was regarded as positive. *Number positive/number tested. #The range of HI titers in the seal serum samples which showed positive by this HI test. — indicates a titer of less than 64. (These tables were taken from K. Ohishi et al. 2002 by obtaining permission.)

virus but also for the control the pandemic influenza. To examine wider distribution of influenza virus in marine mammals, epidemiological survey of other species of seals and cetaceans are now undergoing. Although we could not isolate influenza virus from Caspian seals in our recent study, virus isolation should be essential to understand the ecology and pathogenecity of the virus. The Caspian seal population is reported to decline from about 1 million animals early in the 20th century to 360,000–400,000 by the end of the 1980s (Krylov 1990). Mass-die-off of Caspian seals by morbillivirus infection in 1997 and 2000, further might give damage to this vulnerable species (Forsyth et al. 1998, Kennedy et al. 2000). Continued study of infectious diseases in marine mammals will help to provide an understanding of the cause of mass mortalities and to determine the risks to the survival of marine mammals.

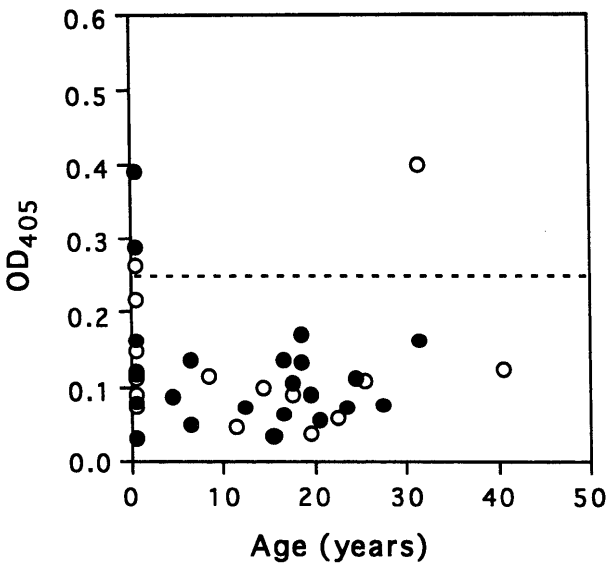

Fig. 4. Antibodies to influenza B virus by ELISA in seal sera collected in the year 2000.
Absorbance at 405 nm versus their ages (years) is shown. B/Lee/40 was used for antigens. See the legends for Fig. 1.

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