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ANTIBODIES AGAINST INFLUENZA VIRUS TYPES A AND B IN CANADIAN SEALS

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ABSTRACT: Influenza viruses have been reported from marine mammals worldwide, particularly in pinnipeds, and have caused mass mortalities of seals in North America and Europe. Because influenza viruses in marine mammals can be zoonotic, our objective was to examine Canadian phocids for exposure to influenza A and B viruses in order to understand health risks to wild populations as well as to humans who consume or handle these animals. Blood was collected from 394 seals in eastern Canada from 1994 to 2005. Sera were screened for exposure to influenza viruses in three resident species of seals: harbour, *Phoca vitulina* (*n*=66); grey, *Halichoerus grypus* (*n*=82); ringed, *Phoca hispida* (*n*=2); and two migrant species: harp, *Pagophilus groenlandica* (*n*=206) and hooded, *Cystophora cristata* (*n*=38). Included were samples from captive grey (*n*=1) and harbour seals (*n*=8) at two aquaria. Sera were prescreened using indirect enzyme-linked immunosorbent assay (ELISA), and antibodies against influenza A virus were confirmed using a commercial competitive ELISA (IDEXX Europe B.V.). A subset of influenza A virus positive sera was used to determine common virus subtypes recognized by sera using reference strains. All positive sera in the indirect ELISA reacted with influenza A virus subtypes H3, H4, and H10 using a hemagglutination inhibition assay. Sera from harbour, grey, harp, and hooded seals had antibodies against influenza A and influenza B viruses (some cross-reactivity occurred). Overall, 33% (128/385) of wild seals were seropositive to influenza viruses, with the highest seroprevalence in harp (42%) followed by harbour (33%), grey (23%), and hooded (11%) seals. Antibodies were detected in both sexes and most age classes of wild seals. Two of eight captive harbour seals were seropositive to influenza B virus and four had cross-reactions to influenza A and B viruses. This study reports antibodies against influenza A and B viruses in four seal species from the same geographic area in eastern Canada.

Key words: Canada, influenza viruses, seals, serology.

INTRODUCTION

Mass mortalities of pinnipeds due to pneumonia associated with influenza virus infection have been reported from North America (Webster et al. 1981a; Anthony et al. 2012) and Europe (Zohari et al. 2014; van den Brand et al. 2016). Epizootics, primarily involving harbour seals (*Phoca vitulina*), have occurred on the east coast of the US due to subtypes H7N7 in 1979–80, H4N5 in 1982–83, H4N6 in 1991, H3N3 in 1991–92, H3N8 in 2011, and on the northeast coast of Europe due to subtype H10N7 in 2014–15. Influenza viruses have been detected serologically or isolated from pinnipeds (Lang et al. 1981; Gulyaeva et al. 2018; Shin et al. 2019), cetaceans (Lvov et al. 1978; Hinshaw et al. 1986; Groth et al. 2014), and northern sea otters (*Enhydra lutris kenyoni*; White et al. 2013; Capuano et al. 2017).

Mass mortalities of marine mammals in which the etiologic agent was not identified include those in harbour seals, harbour porpoises (*Phocoena phocoena*), crabeater seals (*Lobodon carcinophagus*), and grey seals (*Halichoerus grypus*; Bárðarson 1931; Laws and Taylor 1957; Gallagher and Waters 1964; Härkönen et al. 2008). Clinical signs and pathologic lesions in these reports suggest viral pulmonary infections. Influenza A virus infection in seals can cause respiratory distress, nasal discharge, and swollen neck and thorax and, on histologic examination, diffuse hemorrhagic pneumonia with necrotizing bronchitis and bronchiolitis as well as hemorrhagic alveolitis and subcutaneous emphysema of the thorax and neck (Geraci et al. 1982;

Anthony et al. 2012). Pneumonia in marine mammals also may be caused by viruses such as phocine distemper virus (Duignan et al. 2018), phocine herpesvirus-1 (Borst et al. 1986), and by bacteria, parasites, or co-infections. A thorough description of the clinical signs, histopathology, and identification of the etiologic agent is required to confirm a diagnosis (Kennedy-Stoskopf 2001).

Influenza is caused by the RNA viruses influenza A, influenza B, influenza C, and influenza D. Influenza A viruses are widespread in wild aquatic birds, particularly Anseriformes and Charadriiformes, which are the natural reservoirs of avian influenza A viruses. Influenza A viruses may infect other hosts, including mammals, and become adapted to new hosts. Some avian influenza A viruses can become highly pathogenic in poultry and can spill over into domestic and wild animals as well as humans. Influenza B and C viruses are human influenza viruses with no known wildlife reservoir and are not observed in aquatic birds (Krauss et al. 2004). Influenza D viruses are found in livestock (Hause et al. 2014). Influenza A viruses are classified based on their variable surface antigens, hemagglutinin (HA) and neuraminidase (NA) proteins, and at present there are 18 HA (H1–H18) and 11 NA (N1–N11) known.

Influenza A viruses reported in marine mammals are of avian origin (Webster et al. 1981a; Bodewes et al. 2015a). Influenza B virus identified in harbour seals and gray seals in Dutch rehabilitation centers (Osterhaus et al. 2000; Bodewes et al. 2013), in Caspian seals (*Phoca caspica*; Ohishi et al. 2002), and in South American fur seals (*Arctocephalus australis*; Blanc et al. 2009) were circulating in adjacent human populations before identification in seals, as was the pandemic A/H1N1pdm09 virus that spilled into harbour seals, northern elephant seals (*Mirounga angustirostris*), and California sea lions (*Zalophus californianus*; Boyce et al. 2013; Goldstein et al. 2013). Humans became infected with influenza A virus H7N7 from harbour seals. Infections resolved within several days, and there was no seroconversion and no respiratory infections developed, but the zoonotic risk from handling infected marine mammals remains a concern (Duignan et al. 2018).

Seven pinniped species are hunted in Canada: walrus (*Odobenus rosmarus*), bearded seals (*Erignathus barbutus*), ringed seals (*Pusa hispida*), harbour seals, hooded seals (*Cystophora cristata*), harp seals (*Pagophilus groenlandicus*), and grey seals, most for subsistence purposes by Inuit and others, with the latter two species commercially hunted. These pinnipeds have different distributions, abundance, and diet (Kingsley and Byers 1998; Stenson et al. 2006, 2020; Department of Fisheries and Oceans 2017), which affect their exposure to parasites and pathogens. Few studies have examined marine mammals for exposure to influenza viruses in Canadian waters (Geraci et al. 1982, 1984; Nielsen et al. 2001; Puryear et al. 2016). Our study was undertaken to determine exposure to influenza viruses in ringed, harbour, grey, hooded, and harp seals in Canadian waters in order to understand health risks to seals and humans (subsistence and commercial hunters, biologists, veterinarians, and others). Given that rehabilitation of stranded marine mammals, especially pinnipeds, may pose a health risk to humans handling these animals (Hunt et al. 2008), as well as to wild populations when potentially infected animals are released (Measures 2004), we included captive seals at two aquaria.

MATERIALS AND METHODS

Seals (n=377) were live-captured or shot under a scientific permit from Fisheries and Oceans Canada from 1994 to 2005 on the east coast of Canada (Fig. 1). Handling and sampling of all seals was conducted according to protocols and ethical approval by Fisheries and Oceans Canada following guidelines by the Canadian Council of Animal Care (2003, 2014).

Harp seals (n=204) were sampled in March 1997 and in March 1999–2001 and hooded seals (n=36) in March 1996 and March 2005 near the Magdalen Islands, Quebec (47°82′39″N, 61°85′29″W). One harp seal was sampled in September 1994 off Newfoundland (exact location unknown). Harbor seals (n=54) were sampled at

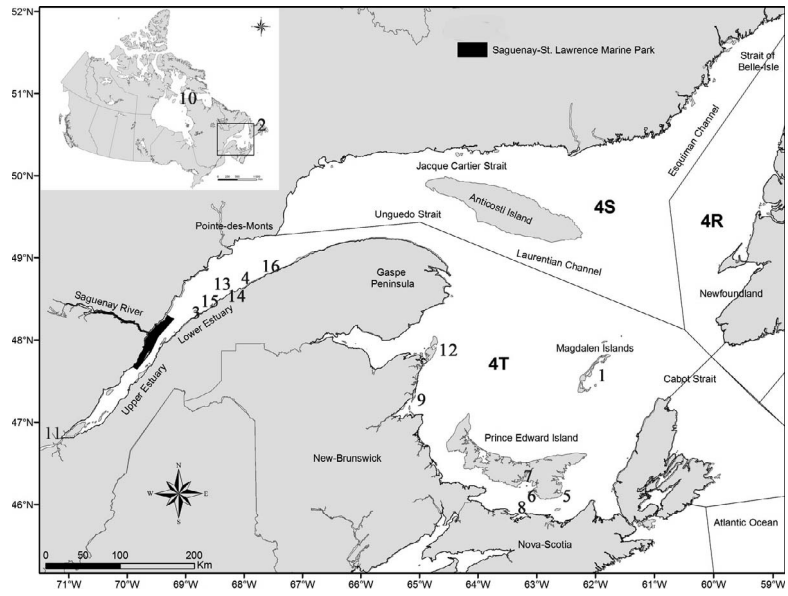

FIGURE 1. Map of eastern Canada showing 16 locations where seals were sampled. 1. Magdalen Islands, Quebec. 2. Newfoundland. 3. Bic, Quebec. 4. Métis-sur-Mer, Quebec. 5. Murray Harbour, Prince Edward Island. 6. Governor's Island, Prince Edward Island. 7. Charlottetown, Prince Edward Island. 8. Amet Island, New Brunswick. 9. Portage Island, New Brunswick. 10. Salluit, Quebec. 11. Ste. Foy, Quebec. 12. Shippagan, New Brunswick. 13. Ste-Luce, Quebec. 14. Ste-Flavie, Quebec. 15. Pointe-au-Père, Quebec. 16. Petit-Matane, Quebec.

Bic, Quebec (48°82′39″N, 68°85′39″W) from May to August 1995; at Métis-sur-Mer, Quebec (48°40′N, 68°00′W) in July 1996; at Murray Harbour, Prince Edward Island (46°0′24″N, 62°31′32″W); on Governors Island, Prince Edward Island (46°8′9″N, 63°3′32″W) in September 2000; and near Charlottetown, Prince Edward Island (46°14′24″N, 63°8′23″W) in October 2001. Grey seals (n=80) were sampled on Amet Island, New Brunswick (45°50′N, 63°10′W) in January, February, June, and September 2000, and in February, March, and October 2001, on Governors Island, Prince Edward Island in September 2000; on Portage Island, Miramichi Bay, New Brunswick (47°7′N, 65°10′W) in June and October 2000; and near Charlottetown, Prince Edward Island in January and February 2000 and October 2001. Two ringed seals were sampled from a subsistence hunt in Salluit, Quebec (62°13′N, 75°39′W) in September 1999. Eight harbour seals and one grey seal held permanently at Aquarium du Québec, Ste-Foy, Quebec (46°45′3″N, 71°17′18″W) and New Brunswick Aquarium and Marine Centre, Shippagan, New Brunswick (47°44′38″N, 64°43′4″W) were also sampled in November and December 1996. Eight seals stranded alive or dead were also sampled: one harp seal at Ste-Luce, Quebec (48°33′32″N, 68°19′49″W) in December 2005; one grey seal at Ste-Flavie, Quebec (48°36′23″N, 68°14′28″W) in July 2005; two hooded seals at Pointe-au-Père, Quebec (48°30′51″N, 68°27′53″W) in December 1996 and November 2004; and four harbor seals at Bic on 22 July 1995, Métis-sur-Mer on 22 July and 28 July 1995 and at Petit-Matane, Quebec (48°51′N, 67°26′W) on 21 June 2001.

Blood was obtained from a jugular vein or the heart of shot seals or a lumbar intravertebral extradural vein of live-captured seals restrained in a capture pole-net, using 50–152-mm 18-ga needles and syringes, and transferred to untreated Vacutainers® (Becton Dickinson and Company, Franklin Lakes, New Jersey, USA). Blood samples were handled as described (Bellehumeur et al. 2016) and sera stored at –80 C until analysis.

Seals were aged by counting the number of growth layer groups (GLGs) in the dentine or cementum of lower canine or incisor teeth extracted from the lower jaws of seals; one GLG=1 yr of age. Seals were classified as young-of-the-year (YOY) seals or pups in their first year of life or age <1 yr; juveniles were age ≥1 yr and sexually immature; or adults, which were sexually mature as described (Measures et al. 2004). One grey, one harbour, two ringed, and two hooded seals were aged as juveniles or adults based on standard nose-to-tail length or weight.

Seal sera were first prescreened for antibodies against influenza A and B viruses by indirect enzyme-linked immunosorbent assay (ELISA) as described (Osterhaus et al. 2000; see Supplementary Material). The influenza A and influenza B indirect ELISA tests were performed separately. The optical density (OD) was measured at 450 nm. The cut-off value (OD from which the OD of the bovine serum albumin control was subtracted) was set at 0.9 based on positive and negative control ferret sera. In parallel, we used a commercial competition ELISA (IDEXX Europe B.V., Hoofddorp, the Netherlands) for the detection of influenza A antibodies as recommended by the manufacturer. All seal serum samples were tested twice, and test results were considered positive if one or both tests were positive. An equivalent test was not available for influenza B virus.

All sera positive in the indirect ELISA or IDEXX assay were tested in hemagglutination inhibition (HI) assays. Sera were incubated with the receptor-destroying enzyme (RDE) *Vibrio cholerae* neuraminidase at 37 C overnight, followed by incubation at 56 C for 1 h to inactivate the RDE. Nonspecific agglutination was not observed after this RDE treatment. Serial twofold serum dilutions (50 μL) starting at a 1:10 dilution were incubated with 4 hemagglutinating units of virus (25 μL) at 37 C for 30 min. Then 25 μL of 1% turkey red blood cells were added and incubated for 1 h at 4 C. The HI titer was measured as the reciprocal of the highest serum dilution that completely inhibited hemagglutination. A subset of influenza A virus-positive seal sera for which sufficient serum volumes were available (n=42) was used to determine the common virus subtypes that were recognized by the seal sera at serial dilutions ranging from 1:50 to 1:400, using several reference strains (see Supplementary Material). Seal sera reacted only with H3, H4, and H10; all ELISA-positive seal sera for which smaller serum volumes were available were subsequently tested using final titrations starting at 1:20 with this panel. An HI titer of 1:20 or higher was considered positive. The relationship between the three serologic assays is shown in Figure 2.

Statistical analyses to detect differences in seroprevalence (n seropositive/n tested ×100) by species, sex, age class, or number of GLGs or sampling date were analyzed using Sigmaplot software, version 13.0 (SYSTAT, Palo Alto, California, USA).

RESULTS

Of the wild (noncaptive) seals, 33.2% (128/385) (95% confidence interval: 29.6–36.4%) were seropositive to influenza A or B viruses. Seroprevalence was significantly higher in harp seals (χ^2=19.93, P<0.001) than in harbour, grey, and hooded seals; ringed seals were seronegative (Table 1). There was no significant difference in seroprevalence by sex or age for each wild species except for harp seals (Table 2). Adult harp seals had a significantly greater seroprevalence (χ^2=30.65, P<0.001 using age class or χ^2=58.23, P<0.001 using number of GLGs) than did juvenile or YOY harp seals (seals with unknown age [n=26] excluded). Seroprevalence in adult harp seals tended to decrease with increasing age (GLGs; Pearson's correlation= –0.212, P=0.0254).

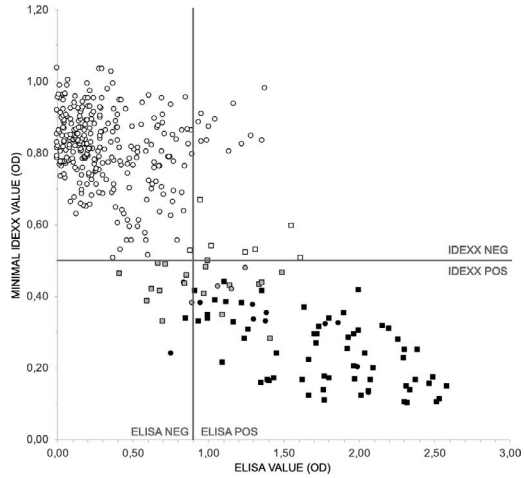

FIGURE 2. Relationship between the results of three serologic assays for influenza A virus testing in seals. Shown are the enzyme-linked immunosorbent assay (ELISA) optical density (OD) data plotted against the lowest OD value of two IDEXX test results, with positive hemagglutination inhibition (HI) data marked. Open symbols represent IDEXX negative samples, gray symbols represent IDEXX single-positive tests, black symbols represent IDEXX double-positive tests, and square symbols are HI-positive samples. All ELISA-positive and IDEXX-positive, and ELISA/IDEXX-positive, samples were titrated in an HI assay. The lower right quadrant shows ELISA-positive, IDEXX-positive samples. High titers in ELISA generally correspond to double-positive IDEXX test results and measurable HI.

Three mother-pup pairs of harp seals were seropositive; pups were a few days old and still nursing. Six YOY harbour seals sampled in September or October (2 mo postweaning)

TABLE 1. Number of enzyme-linked immunosorbent assay seropositive seals from the east coast of Canada sampled from 1994 to 2005 to detect exposure to influenza viruses.

Species	No. sera positive/ number of sera tested	% Seropositive (seroprevalence) with (95% confidence interval)[a]
Ringed seal (*Phoca hispida*)	0/2	0
Hooded seal (*Cystophora cristata*)	4/38[b]	10.5 (2.9–24.9)
Grey seal (*Halichoerus grypus*)		
Wild	19/81[c]	23.4 (14.9–34.2)
Captive	0/1	0
Total	19/82	23.2 (NC)
Harbour seal (*Phoca vitulina*)		
Wild	19/58[d]	32.8 (21.3–46.3)
Captive	6/8	75 (NC)
Total	25/66	37.9 (NC)
Harp seal (*Pagophilus groenlandica*)	86/206[e]	41.7 (34.4–49.1)
Total		
Wild	128/385	33.2 (29.6–36.4)
Captive	6/9	66.7 (NC)
Total	134/394	34.0 (NC)

[a] NC = confidence interval not calculated.
[b] Includes two stranded hooded seals, one of which was seropositive.
[c] Includes one stranded seropositive grey seal.
[d] Includes four stranded seronegative harbour seals.
[e] Includes one seronegative stranded harp seal.

and two YOY grey seals sampled in September (7 mo postweaning) were seropositive. Monthly sample sizes stratified by age class were small, but there was a significant difference in seroprevalence by month for all age classes of harbour seals (χ^2=22.94, P<0.001), with more harbour seals seropositive in September or October (Fig. 3). The longest sampling time series was for harp seals, in which there was a significant difference in seroprevalence by year of sampling (χ^2=49.51, P<0.001), with the highest seroprevalence in 1999 and 2000 (Fig. 4).

Antibodies against influenza A virus appeared more prevalent than did antibodies against influenza B virus in wild seals except in harbour seals, in which antibodies against influenza B virus predominated (Table 3). The

TABLE 2. Number of enzyme-linked immunosorbent assay seropositive seals from the east coast of Canada, sampled from 1994 to 2005, against influenza viruses by age and sex classes (number positive/number tested). YOY = young of the year.

Species	Adult male	Adult female	Juvenile male	Juvenile female	YOY male	YOY female	Unknown age and/or sex	Total
Ringed seal (*Phoca hispida*)	0	0	0/2	0	0	0	0	0/2
Hooded seal (*Cystophora cristata*)	1/5	3/17	0	0	0/9	0/7	0	4/38
Grey seal (*Halichoerus grypus*)	6/16	9/43[a]	0/4	1/7	2/3	0/8	1/1	19/82
Harbour seal (*Phoca vitulina*)	5/7[b]	4/10[c]	6/15	4/9	3/14	3/11[d]	0	25/66
Harp seal (*Pagophilus groenlandica*)	5/6	58/114	0/1	0	2/26	4/32	17/27	86/206
Totals	17/34	74/184	6/22	5/16	7/52	7/58	18/28	134/394

[a] Includes one captive seronegative.
[b] Includes three captive seropositives.
[c] Includes three captive seropositives and 1 seronegative.
[d] Includes one captive seronegative.

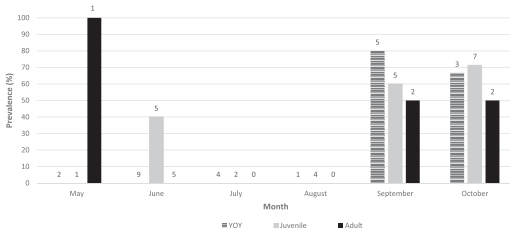

FIGURE 3. Prevalence of antibodies to influenza viruses in wild harbor seals from the east coast of Canada (excludes captive seals) by age class (young-of-the-year, juvenile, adult) sampled from May to October 1994–2005. Sample sizes are indicated above bars or x-axis for each month, 0 denotes no sample.

105 wild seal sera seropositive to influenza A were subtyped against a reference panel of avian influenza A viruses: H3 predominated (85/105, 81%), followed by H10 (16/105, 15%) and H4 (4/105, 4%). Of the three H3 viruses in the reference panel, the highest reactivity was seen with an immunogenic 1968 H3, followed by a less immunogenic avian H3, and with no reaction to a seasonal epidemic H3 from humans; thus we conclude that seal sera reacted with avian, not human H3. Excluding cross-reactive seal sera (n=14), antibodies against influenza B virus were detected in 15 seal sera by ELISA but HI titers were <10 against all three influenza B viruses tested; hence the seal-infecting virus lineage remains obscure. Antibodies against influenza A virus subtypes H3, H4, and H10 were identified in harp seals and antibodies against influenza A virus subtypes H3 and H10 in grey seals. Grey and harp seals had the highest HI titers to influenza A virus, up to 1920 and 1280, respectively. Influenza A H3 antibodies in harp seals were particularly prevalent in 1999, 2001, and 2005 (data not shown). Influenza A (H4) antibody was detected only in harp seals; two seals in 1999 and one seal each in 2001 and 2003.

Both aquaria housed seals seropositive to influenza viruses. Two harbour seals (12-yr-old and 17-yr-old females) were seropositive

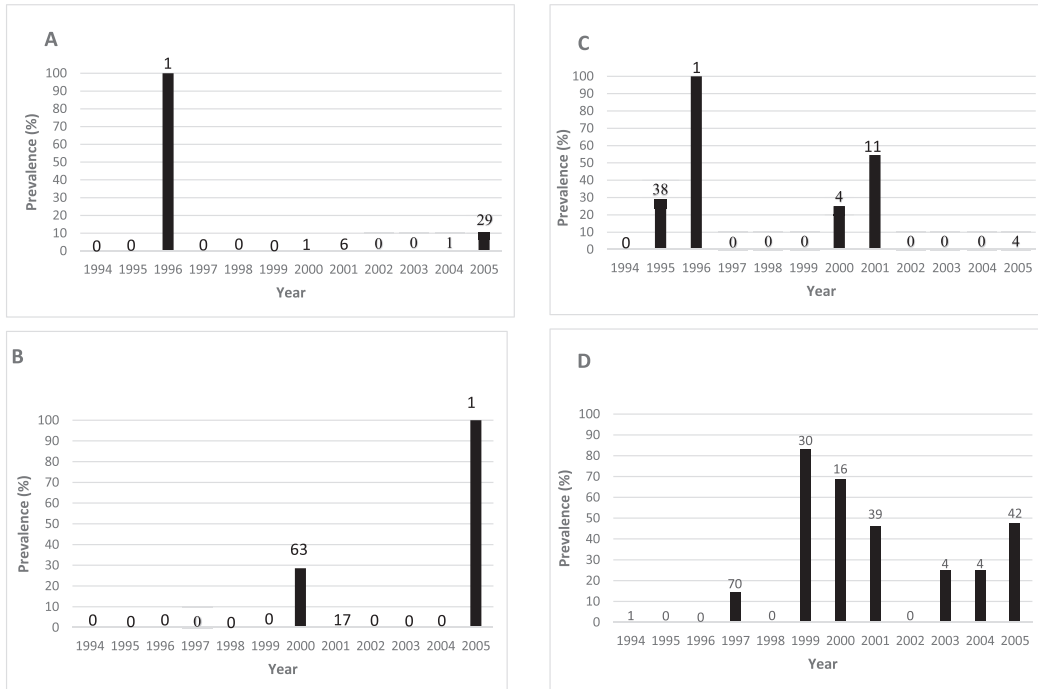

FIGURE 4. Prevalence of antibodies to influenza viruses in wild seals from the east coast of Canada (excludes captive seals) by year sampled from 1994–2005. Sample sizes are indicated above bars or x-axis for each year, 0 denotes no sample. A = hooded seals (*Cystophora cristata*); B = grey seals (*Halichoerus grypus*); C = harbour seals (*Phoca vitulina*); D = harp seals (*Pagophilus groenlandica*).

TABLE 3. Number of influenza virus A subtypes and influenza B detected in enzyme-linked immunosorbent assay seropositive wild and captive seals from the east coast of Canada with hemagglutination inhibition assay positive titers. Some seals were seropositive to both viruses and more than one subtype.

Species	Identified influenza A subtypes			Influenza A (untyped)	Influenza B (untyped)	Influenza A and B cross-reactive
	H3	H4	H10			
Hooded seal (*Cystophora cristata*)	0	0	0	1	0	3
Grey seal (*Halichoerus grypus*)	13	0	5	3	3	0
Harbour seal (*Phoca vitulina*)	0	0	0	3	11[a]	11[b]
Harp seal (*Pagophilus groenlandica*)	72	4	11	12	1	0
Total	85	4	16	19	15	14

[a] Includes two captive seals.

[b] Includes four captive seals.

to influenza B virus and four harbour seals (7-, 12-, and 13-yr-old males and a 22-yr-old female) had cross-reactions against influenza A and B viruses (Table 3). Two harbour seals (27-yr-old female and 7-mo- old female) and one grey seal (12-yr-old adult female) were seronegative. Of the four seals born in captivity, two (7-yr-old male and 17-yr-old female harbour seal) were seropositive and two (12-yr-old female grey seal and 7-mo-old female harbour seal) were seronegative. Four wild-caught harbour seals were seropositive (12–22 yr old, two males and two females); one seronegative harbour seal (27-yr-old female) had an unknown history.

DISCUSSION

Four species of Canadian seals within the St. Lawrence ecosystem in our study were exposed to influenza A and B viruses. Seroprevalence of influenza A virus was relatively high in harp (42%) and harbour seals (33%) and lower in grey (23%) and hooded seals (11%). Live-captured seals and those sampled on whelping patches appeared healthy. One live-stranded grey seal was severely emaciated (80 kg with little subcutaneous fat). This 37-yr-old female was euthanized and at necropsy numerous multiple organ lesions were observed. Histopathologic analyses revealed no lesions consistent with viral infections; many lesions were of a geriatric nature. She was seropositive to influenza A H3 with a HI titer of 320.

Although our two ringed seals were seronegative, 2.5% of 903 ringed seals were seropositive to influenza A virus (subtype not determined) in the Canadian Arctic (Nielsen et al. 2001). Danner et al. (1998) reported one of 32 ringed seals seropositive to influenza A virus (H3 and H7) in Alaska.

The low seroprevalence of influenza A virus in our sample of hooded seals has also been observed in harp and hooded seals from the Barents Sea and north of Jan Mayen in the Greenland Sea (Stuen et al. 1994): 0–11% for hooded seals and 10–27% for harp seals depending on year and location. Hooded seals in the Northwest Atlantic form a panmictic population (Coltman et al. 2007), and low seroprevalence in this population may indicate a low exposure to infected aquatic birds or that hooded seals are less susceptible to infection. We found higher seroprevalence (42%) in Canadian harp seals, which appear reproductively isolated from harp seals in the Barents Sea or those north of Jan Mayen (Perry et al. 2000), although immature seals may wander between these locations (Sergeant 1991). Bogomolni et al. (2008) isolated and identified influenza A virus H3N8 in one by-caught harp seal in New England.

We observed no effect of age or sex with seroprevalence of influenza A virus except for harp seals: seroprevalence was greater in adult harp seals than in juveniles or pups and declined with increasing age among adults. Three seropositive harp seal pups a few days old probably obtained antibodies from their

matched seropositive mothers. In late summer, six weaned YOY harbour seals and two YOY grey seals had low HI titers to influenza virus, indicating either recent exposure or retained maternal antibodies. Puryear et al. (2016) isolated influenza A virus in 9% of healthy weaned grey seal pups and detected antibodies to influenza A virus in 19% of pups in Cape Cod, Massachusetts (these pups were at least 4 wk old). It is unknown how long seals retain antibodies against influenza viruses and whether antibodies are protective against subsequent infection. Maternal antibodies against influenza B virus declined to undetectable levels in harbour and grey seals greater than 6 mo old in the Netherlands (Bodewes et al. 2013). In contrast, Bodewes et al. (2015b) observed no decline in antibodies against influenza A H10N7 in harbour seals up to 156 d.

Seroprevalence varied annually in all four seropositive species of seals, particularly in harp seals with a seroprevalence >60% in two consecutive years (1999 and 2000). While antibodies against influenza A virus subtypes H3 and H10 were detected in grey and harp seals in most years, often concurrently, H4 was detected only in harp seals and infrequently. Importantly, as subtypes H3 and H4—and to a lesser extent also H10—are antigenically related and H3 HI antibody titers were generally much higher than those against H4 and H10, it cannot be excluded that H4 and H10 serum reactivity was based on cross-reactivity due to H3-specific antibodies (see Latorre-Margalef et al. 2013).

Our generally good agreement between the three assays used (indirect ELISA, commercial competitive ELISA, and HI assay) indicates that serologic results were robust and reproducible (Fig. 2). Negative HI results were unlikely due to antigenic variation in avian HA, as these subtypes are antigenically similar globally. However, for influenza B positive sera, antigenic drift may account for negative HI results.

Interannual variability in viral shedding and seroprevalence of influenza A virus has been observed in grey seals from Sable Island, Nova Scotia (Puryear et al. 2016). Interannual periodicity of some influenza A subtypes has been reported in ducks (Hinshaw et al. 1980; Krauss et al. 2004) and in shorebirds (Bahnson et al. 2018) and is considered common (Krauss and Webster 2010). A diversity of subtypes have been identified in adult grey seals from Sable Island (H3N8, H2N3, H3N8, H4N6, H6, H8N4, H13N6, H16N2) and these plus additional subtypes (pH1N1, H7N3, H11N9) in pups and adults from Cape Cod (Puryear et al. 2016). Influenza A subtypes H3, H4, and H10 were involved in mass mortalities of seals in the eastern US and northeastern Europe.

Geraci et al. (1982, 1984) examined wild Canadian seals in 1981 for evidence of influenza A virus infection in light of mass mortalities of harbour seals off New England in 1979–80. Only three adult males of the 99 grey seals (77 adults, 22 1-mo-old pups) sampled on Sable Island had antibodies to influenza A/seal/Mass/1/80 (H7N7); no virus was isolated nor were there any clinical or gross lesions indicative of lung disease in these seals. They also examined harbour, harp, and hooded seals from eastern Canada; none were seropositive or showed evidence of lung disease. Puryear et al. (2016) reported 50% of 20 adult grey seals sampled in summer 2013 on Sable Island were seropositive to influenza A virus (see above reported subtypes) with less than 10% shedding virus.

Seroprevalence of influenza viruses in harbour seals was relatively high (33%), including in captive harbour seals (some born in captivity), with sera reactive to influenza B or cross-reactive to influenza A and B viruses. No mass mortalities of harbour seals due to influenza viral infections have been reported in Canadian waters. Experimental studies with seals exposed to influenza A (H7N7, H4N7) failed to reproduce severe viral pneumonia as observed during epizootics (Webster et al. 1981a; Geraci et al. 1984; Hinshaw et al. 1984). Dense aggregations of seals, unusual environmental conditions, and secondary infections may play a role in severity of respiratory disease associated with influenza virus infections (Geraci et al. 1982; Callan et al. 1995; van den Brand et al. 2016). In the St.

Lawrence Estuary (SLE), frequent human interactions with harbour seal pups occur annually in near-shore environments when females leave their pups alone to forage and people think these pups are abandoned (L.N.M. pers. obs.). This leads to needless human contact, handling, and unnecessary rehabilitation of pups, and occurs frequently in North America and Europe, risking exposure to infectious diseases (Measures 2004).

Seal species vary in their habitat and breeding biology. Harbour seals (resident in the SLE and Gulf of St. Lawrence [GSL]) breed on intertidal ledges, sand bars, and islands May–June and are associated with coastal or near shore environments; grey seals (resident in the SLE and GSL) breed on islands or ice December–January and are generally associated with coastal environments; and hooded seals and harp seals (seasonal migrants from the Arctic) breed on ice in March in the GSL and are typically found offshore. Hooded seals spend less time nursing a pup (4 d) on the whelping patch compared to harp seals (12 d), grey seals (17 d), or harbour seals (30 d) on average (Reeves et al. 1992). Increased duration on the whelping patch may expose seals to avian hosts infected with influenza viruses, such as gulls (Laridae), which scavenge placentas and dead pups.

The identified subtypes in our study (H3, H4, H10) have been reported from Anseriformes and Charadriiformes, including from the North Atlantic (Olsen et al. 2006; Wille et al. 2014; Hall et al. 2015; Lang et al. 2016). Within the GSL and SLE are inshore and offshore marine birds, many breeding in large coastal or island colonies as breeding migrants. These include larids, alcids, cormorants, Northern Gannet (*Morus bassanus*), and Leach's Storm Petrel (*Oceanodroma leucorhoa*; Cairns et al. 1991) as well as sea ducks (Gauthier and Aubry 1996). While HA subtypes H1–H16 and NA subtypes 1–9 are reported from ducks and shorebirds including gulls, H3 and H4 predominate in ducks (Webster et al. 2007) and H13 and H16 are associated with gulls (Krauss and Webster 2010). A great diversity of influenza A viruses have been isolated and subtyped genetically as H3, H4, and H10 from alcids such as Common Murres (*Uria aalge*) and Thick-billed Murres (*Uria lomvia*; Lang et al. 2016). Seals are probably exposed to influenza A viruses via contaminated water (fecal-oral route) when birds shedding virus defecate on reefs, islands, or ice shared with seals. Seals also predate on aquatic birds such as murres (Lucas and McLaren 1988) and ducks (Tallman and Sullivan 2004). Additionally, the degree of social contact among seals may affect transmission of influenza viruses. Harp seals are gregarious, forming large aggregations during whelping or moulting, and grey seals are also gregarious, while harbour seals tend to form small aggregations and hooded seals are more solitary.

Various zoonotic infections (redefined by the WHO 1967; also see Messenger et al. 2014), including respiratory infections, have been reported by marine mammal handlers in rehabilitation centers (Webster et al. 1981b; Hunt et al. 2008). Caution should be exercised in handling seals, alive or dead, and they should not be handled by humans infected with influenza A or B (Measures 2004). Personal protective equipment should be worn by personnel handling any marine mammal in close proximity, such as in rehabilitation centers or zoologic parks, in order to protect against infectious respiratory aerosols—especially as asymptomatic seals may shed virus (Goldstein et al. 2013; Puryear et al. 2016).

Influenza B virus has been reported in harbour seals in a rehabilitation center (Osterhaus 2000; Bodewes et al. 2013), but it is unknown whether the virus was originally transmitted to seals during rehabilitation and then to wild seal populations postrelease or from human interactions with seals on haul-out sites; influenza B circulated in the human population prior to detection in rehabilitating seals. Influenza B predominated in harbour seals examined in eastern Canada and in captive harbour seals in two aquaria, one of which released rehabilitated seals into the SLE for about 8 yr (Measures 2004). Serologic studies suggested that influenza A virus

(including pandemic AH1N1pdm09) and influenza B viruses circulating in nearby human populations spilled into seals and sea lions (Ohishi et al. 2002, 2004; Blanc et al. 2009; Boyce et al. 2013). That zoonotic disease, such as influenza, can be transmitted from humans to animals including wildlife, demands greater awareness of the consequences of human activities (Measures 2004; Messenger et al. 2014). Further research on such diseases will benefit humans, domestic animals, wildlife, and especially endangered species.

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SUPPLEMENTARY MATERIAL

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Supplementary materials for Journal of Wildlife Diseases DOI: 10.7589/JWD-D-20-00175: Lena N. Measures and Ron A.M. Fouchier. ANTIBODIES AGAINST INFLUENZA VIRUS TYPES A AND B IN CANADIAN SEALS

Materials and Methods

Sera collected from 394 seals, including harbour seals (*Phoca vitulina*), grey seals (*Halichoerus grypus*), ringed seals (*Phoca hispida*), harp seals (*Pagophilus groenlandica*) and hooded seals (*Cystophora cristata*), from eastern Canada, 1994 to 2005, were tested using an indirect ELISA as described (Osterhaus et al. 2000). Purified A/Netherlands/18/94 or B/Shanghai/361/02 antigens were diluted 10-fold in 0.1% triton X100/carbonate buffer, pH 9.6 and 100 µL antigen was coated in each well of a 96-well plate at 4 C overnight. Bovine serum albumin (BSA) (100 uL, 0.002%) was coated as negative control. Blocking was performed with PBS, 2% BSA for 1 h at 37 C, and the plates were washed with PBS, 0.05% tween-20. Fifty µL of 1:50 diluted seal serum in PBS, 5% NaCl, 2% BSA, 0.1% ELK was added and incubated at 37 C for 1 h. The plates were washed with PBS, 0.05% tween-20 and 50 µL 1:1000 diluted peroxidase labelled Protein A in PBS, 5% NaCl, 2% BSA, 0.1% ELK was added and incubated for 1 h at 37 C. The plates were washed with PBS, 0.05% tween-20 and 50 µL substrate solution (TMB) was added. After 10 min incubation at room temperature in the dark, 50 µL 1M H_2SO_4 was added.

Reference strains used to test a subset of influenza A virus positive seal sera for which sufficient sera was available were: A/Teal/Netherlands/10/00 (H1N1), A/White-fronted goose/Netherlands/2/99 (H2N2), A/Teal/Netherlands/7/00 (H3N8), A/Mallard/Netherlands/1/99 (H4N6), A/Mallard/Netherlands/3/99 (H5N2), A/Mallard/Netherlands/1/02 (H6N1), A/Mallard/Netherlands/12/00 (H7N3), A/Mallard/Sweden/24/02 (H8N4), A/Mallard/Netherlands/1/05 (H9N2), A/Mallard/Sweden/97/05 (H10N8), A/Shoveler/Netherlands/18/99 (H11N9), A/Mallard/Sweden/86/03 (H12N5), A/BHG/Netherlands/1/00 (H13N8), A/Mallard/Gurjev/263/82 (H14N5), A/Shearwater/W-Australia/2576/79 (H15N9), A/BHG/Sweden/2/99 (H16N3). Viruses A/Teal/Netherlands/7/00 (H3N8), A/Bilthoven/16190/68 (H3N2), A/Netherlands/233/83 (H3N2), A/Mallard/Netherlands/1/99 (H4N6), A/Mallard/Sweden/97/05 (H10N8), B/Harbin/7/94, B/Seal/Netherlands/1/99 and B/Shanghai/361/02