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ROTAVIRUS INFECTIONS IN GALAPAGOS SEA LIONS

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ABSTRACT: Group A rotaviruses infect and cause diarrhea in the young of a broad range of terrestrial mammals, but it is unknown, to our knowledge, whether they infect marine mammals. During February and March of 2002 and 2003, we collected 125 serum samples and 18 rectal swab samples from Galapagos sea lion pups (GSL, *Zalophus wollebaeki*), and 22 serum samples from Galapagos fur seal pups (GFS, *Arctocephalus galapagoensis*) from nine islands of the Galapagos archipelago, Ecuador. Sera were tested for antibodies (immunoglobulin G [IgG]) to rotavirus by an enzyme immunoassay using rhesus rotavirus as the capture antigen. In addition, rectal swabs were analyzed for the presence of rotavirus genomic double-stranded RNA by silver-stained polyacrylamide gel electrophoresis. Antibodies to rotavirus were detected in 27 GSL pups (22%) and five GFS pups (23%), and rotavirus RNA was detected in the fecal sample from one GSL pup (6%). These results provide the first evidence that rotavirus infections are prevalent at an early age in Galapagos sea lions and Galapagos fur seals.

Key words: *Arctocephalus galapagoensis*, Galapagos fur seal, Galapagos sea lion, pinniped, rotavirus, *Zalophus wollebaeki*.

INTRODUCTION

Group A rotavirus infections are common in terrestrial mammalian species, but, to our knowledge, it is unknown whether they infect marine mammals. Severe diarrhea is often associated with initial infection, and adult infections are usually asymptomatic (Kapikian et al., 2001). Seven groups (A–G) exist within the genus *Rotavirus*; viruses in these groups are morphologically identical but serologically unrelated (Kapikian et al., 2001), and all have a segmented double-stranded RNA (dsRNA) genome of 11 segments (Estes, 2001). Rotaviruses have a triple-layered structure of six proteins: VP4 and VP7 form the outer layer, where serotype specificity reside; VP6 forms the intermediate layer and is the major group-specific antigen; and VP2 forms the inner layer that contains the genome in association with the minor proteins VP1 and VP3 and is also a group-specific antigen (Estes, 2001; Kapikian et al., 2001). The outer layer of the virion can be easily removed with chelating agents, like ethylenediaminetetraacetic acid (EDTA), thereby exposing the major group-determining antigen.

The population of Galapagos sea lions (GSLs, *Zalophus wollebaeki*) is declining from approximately 40,000 in 1977–78 to 14,000–16,000 in 2001 (Salazar, 2003; Alava and Salazar, 2006). In contrast, the sister population of California sea lions (CSLs; *Zalophus californianus*) has been steadily increasing during the same time span (Lowry, and Forney, 2005). Most of the GSL decline has been attributed to the 1997–98 and 1982–83 El Niño events, but other causes, like the 2001 Jessica oil spill, have also caused mortality (Salazar, 2003). There are reports of infectious agents that affect the health of GSLs, like eye infections with the parasite *Philophthalmus zalophi* (Dailey et al., 2005); however such studies are limited. Similarly, the population of Galapagos fur seals (GFSs) has declined from 30,000–40,000 in 1977–78 to 6,000–8,000 in 2001 (Alava and Salazar, 2006). Because group A rotaviruses can cause significant morbidity and, in some cases, mortality in humans and

TABLE 1. Locations surveyed in the Galapagos archipelago, Ecuador, for sera from two pinniped species.

Species	Year	Island	Colony	Latitude/longitude	No. of samples
Z. wollebaeki[a]	2002	San Cristóbal	Zona Naval	0°54′S, 89°37′W	7
			La Lobería	0°56′S, 89°33′W	9
			Isla Lobos	0°51′S, 89°34′W	16
		Santa Fe	Bahía Santa Fé	0°48′S, 90°05′W	10
		Santa Cruz	Caamaño	0°45′S, 90°17′W	11
		Seymour	Mosquera	0°24′S, 90°17′ W	9
	2003	Fernandina	Punta Espinosa	0°16′S, 91°27′W	8
		Floreana	Post Office	1°14′S, 90°27′W	9
		Española	Gardner Islet	1°22′S, 89°39′W	7
			Punta Suárez	1°22′S, 89°44′W	10
		San Cristóbal	Zona Naval	0°54′S, 89°37′W	10
			Isla Lobos	0°51′S, 89°34′W	9
		Santa Cruz	Caamaño	0°45′S, 90°17′W	10
A. galapagoensis[b]	2002	Santiago	Puerto Egas	0°15′S, 90°52′W	5
	2003	Isabela	Punta Vicente Roca	0°01′S, 91°29′W	9
		Fernandina	Cape Hammond	0°28′S, 91°37′W	8

[a] Galapagos sea lion (GSL).

[b] Galapagos fur seal (GFS).

several terrestrial animal species, it is of interest to determine whether marine mammals, and GSLs in particular, are also infected with these viruses. The objective of this study was to test GSL and GFS pups for serologic and molecular evidence of rotavirus infection.

MATERIALS AND METHODS

Serum and rectal swab samples

Serum samples were collected from GSL ($n=125$) and GFS ($n=22$) pups on nine islands of the Galapagos archipelago during February and March 2002 ($n=67$) and 2003 ($n=80$; Table 1). The geographic locations surveyed (latitude and longitude) are defined in Table 1. Samples were collected from the jugular veins of animals anesthetized with isoflurane (Heath, 1996). In addition, 18 rectal swabs were obtained in March 2003 from GSL pups; serum was also collected from 12 of these animals. Age of the pups (<1 yr) was based on size (total length <100 cm) and weight (<18 kg). Additional serum samples were obtained in 1999 from six adult CSLs kept in captivity in the Mazatlan aquarium (Sinaloa, Mexico). Sera were separated and frozen at −20 C until tested, whereas rectal swabs were kept at −70 C.

Antirotavirus enzyme immunoassay

Sera from GSL and GFS pups, as well as CSL adults, were tested for antibodies to group A rotavirus by an enzyme immunoassay. The antigen was rhesus rotavirus (strain RRV obtained from H. Greenberg, Stanford University, Palo Alto, California). Virus was cultivated in MA104 African green monkey (*Cercopithecus aethiops*) kidney cells as described previously (Pina-Vazquez et al., 2007). Virions from cell lysates were semipurified by ultracentrifugation and were then treated with EDTA to remove their outer layer, thereby exposing the group antigen, as described (Pina-Vazquez et al., 2007). Double-shelled viral particles were concentrated by ultracentrifugation at 150,000 × G in a SW50 rotor (Beckman, Palo Alto, California) and resuspended in 1/600th volume of Tris 50 mM, pH 8.0. Virus at this stage of purification was used as coat antigen in enzyme immunoassays at a 1/100 dilution in phosphate-buffered saline (PBS) containing 0.05% sodium azide (PBS-Az). At this step, and before adding each new reagent, the plates were washed four times with PBS. Plates were blocked with 10% fetal bovine serum (FBS) in PBS-Az. The GSL or GFS sera were diluted 1:50 in PBS-Az 5% FBS and inactivated 30 min at 56 C and then assayed in duplicate in RRV-coated wells, as well as noncoated wells to determine background binding. To detect bound antibodies,

the plates were incubated 1 hr at 37 C with 2 μg/ml protein A–peroxidase in PBS-Az 5% FBS. The plates were then incubated with 120 μg/ml 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 0.38 μl/ml hydrogen peroxide in citrate buffer (110 mM citric acid, 100 mM sodium citrate, pH 4.0), and optical density (OD) at 405 nm was read. Sera were considered positive if the corrected OD (determined as *OD of RRV-coated wells–OD of noncoated wells*) was >0.1 with the OD of the antigen >2 times the OD of the control. As a positive control, ascites fluid of the monoclonal antibody 1E11, diluted 1:500, was used. This monoclonal antibody was developed against VP6, the major capsid antigen, using human rotavirus strain Wa as the antigen and the solid-phase immunoisolation technique (SPIT) as the screening method (Burns et al., 1988).

Competition enzyme immunoassay

Antigen for competition enzyme immunoassay was RRV purified by rate zonal centrifugation as described (Pina-Vazquez et al., 2007), with minor modifications. Briefly, virus lysates were treated with Genetron® 11 (trichlorofluoromethane, Cydsa, S. A. de C.V., San Pedro Garza García, Mexico), and the supernatant was concentrated by ultracentrifugation, resuspending the pellet in TNC (10 mM Tris, pH 8.0, 150 mM NaCl, 2 mM $CaCl_2$). Viral particles were separated by rate zonal centrifugation in a 15–45% sucrose gradient, and the bands, corresponding to double- and triple-layered particles, were concentrated by ultracentrifugation and resuspended in TNC. Several dilutions of purified antigen were mixed with an equal volume of a 1:100 dilution of heat-inactivated adult CSL serum and incubated overnight at 4 C. The serum, preincubated with an equal volume of competing antigen, was then assayed for antirotavirus antibodies as described above.

Rotavirus RNA electrophoresis

Rotavirus RNA electrophoresis was performed as previously described (Herring et al., 1982), with some modifications. Reovirus type 3 strain Dearing (obtained from E. Méndez, Instituto de Biotecnología, Cuernavaca, Morelos, Mexico) was cultivated in mouse (*Mus musculus*) L. cells in Eagle's minimum essential medium (MEM) with 5% FBS. Monolayers of L cells were infected at a multiplicity of three in MEM without serum, and after adsorbing 1 hr the medium was replaced by MEM-1% FBS. The cells were incubated up to 3 days, when cytopathic effect was evident, and harvested by freezing and thawing once. The rectal swabs were resuspended in 800 μl of distilled water. A volume of 200 μl from rectal swabs or cell culture lysates were mixed with 40 μl of disrupting buffer (50 mM Trizma base, pH 6.8, 50 mM EDTA, 50 mM NaCl, 5% sodium dodecyl sulfate), and the nucleic acids were extracted with one volume of water-saturated phenol and one volume of chloroform. After mixing with vortex and centrifugation for 5 min at 12,000 × G, 90 μl of supernatant were mixed with 10 μl of bromophenol blue 0.0175% in glycerol. The samples were run by polyacrylamide gel electrophoresis without detergent (Laemmli, 1970) 1.5-fold the time necessary for the dye to reach the edge of the gel. The gels were fixed 30 min in 10% ethanol, 0.1% acetic acid, and soaked 30 min in 0.18% silver nitrate in water. After briefly rinsing with water, the gel was soaked in 0.8% formaldehyde and 3% NaOH until the bands appear, stopping the reaction with 5% acetic acid.

Statistical analysis

Differences in antibody prevalence were compared using Fisher's exact test when adult CSL were involved and the expected frequencies were low. When sample sizes were adequate (GSL vs. GFS), antibody prevalence was compared using a chi-square test.

RESULTS

We first assessed the sensitivity and specificity of an enzyme immunoassay using rhesus rotavirus antigen devoid of the outer protein layer (exposing the major rotavirus group antigen VP6). A high signal to background ratio was obtained with an anti-VP6 monoclonal antibody, and six adult CSL sera were shown to be positive with this assay (Fig. 1). The signal produced by adult sera could be competed to background levels with rotavirus purified by rate zonal centrifugation (Fig. 2), hence the assay specifically detected antirotavirus antibodies.

Of the 125 GSL sera samples, 27 (22%) were determined to be positive for the presence of antirotavirus IgG. Similarly, five (23%) of 22 GFS sera samples were positive for antirotavirus IgG. The average OD of six CSL adult sera samples was slightly higher than the average OD of

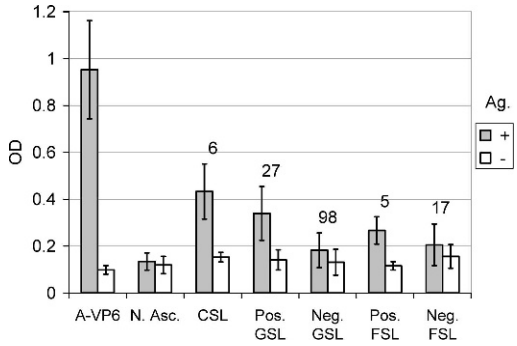

FIGURE 1. Comparison of the average optical density (OD) values of sera from different pinnipeds assayed by antirotavirus enzyme immunoassay. Columns indicate average OD values obtained from wells coated (+) or not (−) with rotavirus antigen from antirotavirus-positive and antirotavirus-negative Galapagos sea lion pups (GSL), antirotavirus-positive and antirotavirus-negative Galapagos fur seal pups (GSF), and adult California sea lions (CSL), all of which were antirotavirus positive. As controls, anti-VP6 monoclonal antibody 1E11 and normal ascites fluid are included. Bars indicate standard deviation of the number of sera indicated above each pair of columns. There was no difference in the ratio of seropositive pups between species (GSL vs. GFS, $P=0.9060$), whereas the ratios of seropositive adults was different from that of pups of both species (CSL vs. GSL, $P=0.0002$; CSL vs. GFS, $P=0.0012$).

these 27 antirotavirus positive sera form GSL pups (Fig. 1) as visualized from the cumulative relative frequency of the corrected OD (Fig 3.). Even though the OD values of adult CSF sera fall within the range of OD values of positive GSL pups, the distribution of values is different, suggesting that adults have higher titers of antirotavirus antibodies. In humans, adults have higher titers of antirotavirus antibodies than infants (Brussow et al., 1988).

The finding of antibody-positive GSL pups prompted us to find animals that shed virus in stools. RNA was extracted from rectal swabs of 18 GSL pups and analyzed by dsRNA electrophoresis. One animal of 18 (6%) was shown to excrete rotavirus (Fig. 4). The RNA pattern of the GSL rotavirus and the prototype stain RRV have the same pattern of four dsRNA groups of segments, 1–4, 5–6, 7–9, and

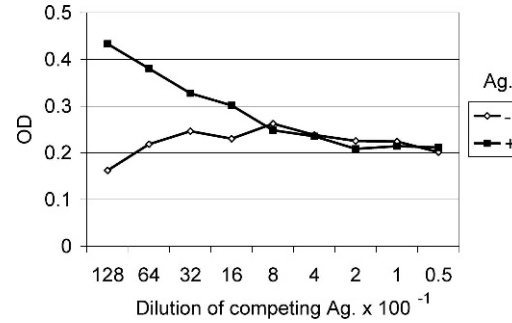

FIGURE 2. Competition antirotavirus enzyme immunoassay of a highly positive adult California sea lion (CSL) serum with several dilutions of purified rotavirus. Average optical density (OD) values obtained from wells coated (+) or not (−) with rotavirus antigen are shown.

10–11, which is easily differentiated from the reovirus pattern of 10 segments (Fig. 4) and unequivocally corresponds to group A rotavirus. There are some differences, however, between the dsRNA pattern of RRV and the GSL rotavirus, in which case segments 5 and 6 comigrate and one of the segments of the group 7–9 had slow migration.

DISCUSSION

We performed a serologic survey of seven of the 13 major islands of the Galapagos archipelago covering the known

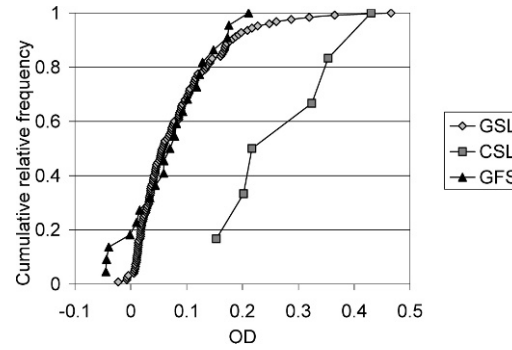

FIGURE 3. Cumulative relative frequency of the corrected optical density (OD) values (*OD of rhesus rotavirus [RRV] coated wells–OD of noncoated wells*) of Galapagos sea lion pups (GSL), Galapagos fur seal pups (GFS), and adult California sea lion (CSL) sera samples, as determined by antirotavirus enzyme immunoassay.

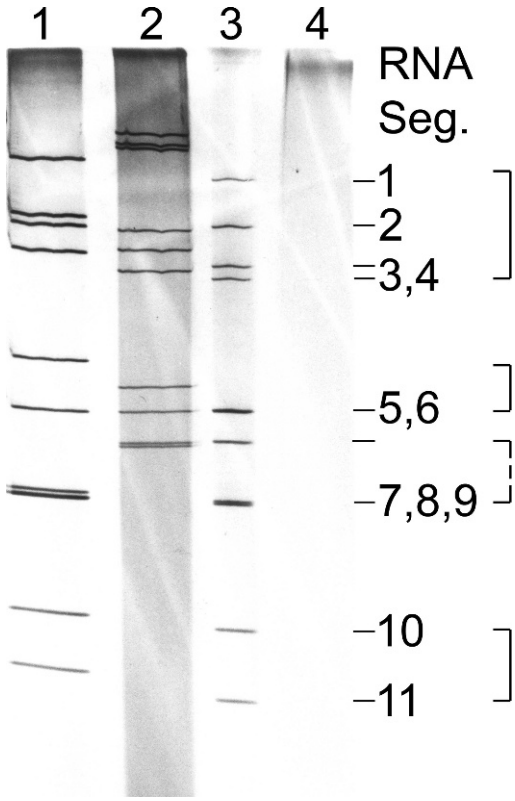

FIGURE 4. Genomic double-stranded (dsRNA) electrophoresis of rotavirus from stools of a Galapagos sea lion (GSL) and as controls prototype group A rotavirus and type 3 reovirus strains were used. RNA was extracted and analyzed by silver-stained polyacrylamide gel electrophoresis of the reference group A rhesus rotavirus (lane 1), reovirus type 3 strain Dearing (lane 2), rotavirus-positive sample from a GSL (lane 3), and one rotavirus-negative sample from a GSL (lane 4). Genomic RNA segment numbering of the GSL rotavirus and the groups of segments are indicated on the right.

geographic range of the GSL. Two additional islands were surveyed to obtain GFS sera, and on Fernandina, both species were surveyed in different rockeries. A high prevalence of antirotavirus antibodies were detected in GSL pups (22%) and an equally high prevalence in GFS pups (23%). This finding indicates that, similar to the findings in terrestrial mammals, group A rotavirus infections in both GSLs and GFSs are acquired at an early age. Adult GSLs were not included in this study; however six adults of the sister species *Z. californianus* were all positive for antirotavirus antibodies and had a distribution of OD values, which suggests that CSL adults have higher antirotavirus titers than GSL or GFS pups. Antirotavirus titers in humans increase with age from multiple asymptomatic infections throughout life (Brussow et al., 1988). It is possible that the mothers of most of the pups included in this study had significant titers of antirotavirus antibodies; however, we considered that there is no transplacental transfer of immunoglobulins in *Z. wollebaeki* or *A. galapagoensis* because, in other pinnipeds, transplacental transfer of immunoglobulins is low or absent (Cavagnolo, and Vedros, 1979; Ross et al., 1994; King et al., 1998), consistent with their endotheliochorial placentation (Dierauf et al., 1986). It is not clear, however, whether neonates of the different pinniped families acquire maternal antibodies via colostrum or milk as suggested for phocids (harbor seal [*Phoca vitulina*] and grey seal [*Halichoerus grypus*]; Ross et al., 1994; Measures et al., 2004). On the other hand, in otariids (Northern fur seal [*Callorhinus ursinus*]), a low rate of IgG appearance and a high rate of IgM appearance after birth in the sera of neonatal pups suggested poor absorption of maternal antibodies (Cavagnolo and Vedros, 1979). Because it is unclear whether GSL and/or GFS pups have maternal antibodies, the detection of rotavirus antibodies cannot be considered as definitive proof of past infection.

We detected excretion of rotavirus in rectal swabs of one (6%) of 18 GSL pups. To our knowledge, this is the first report of a group A rotavirus infecting a marine mammal. The electrophoretic patterns for segments 5 and segments 7, 8, or 9 of the prototype strain RRV and the GSL rotavirus differed. Genomic segment 5 of group A rotaviruses is highly variable in sequence (Matthijnssens et al., 2008) and frequently suffers rearrangements that can modify its size (Desselberger, 1996; Taniguchi et al., 1996). To a lesser extent, segment 7 has also been shown to rearrange to higher molecular weights

(Mendez et al., 1992; Gault et al., 2001). In contrast, segment 6 is more stable in size and sequence (Desselberger, 1996; Matthijnssens et al., 2008). On this basis, we presume that segments 5 and 6 comigrate in the GSL rotavirus, whereas a segment of the 7–9 group may have a higher molecular weight. Sequence analysis of these segments and comparison with more GSL rotaviruses will be necessary to test this hypothesis.

The first exposure of GSL pups to rotavirus infection is in the relatively long, 5-mo nursing period, facilitated by crowding in the rockeries. Persistence of rotavirus in the marine environment is also possible considering that the outer protein layer of the rotavirus particles, which is essential for infectivity, is stabilized by calcium (Estes, 2001). The concentration of calcium in seawater is 10 mM (Müller et al., 1978), well above that necessary to preserve the integrity of the viral outer layer. In one study, the infectivity of RRV was increased by 3.8-fold by raising the basal calcium concentration of the infection media from 1.8 to 10 mM (Pando et al., 2002). Depending on the strain, calcium concentrations from 0.6 to 0.01 mM are sufficiently low to solubilize the outer capsid, thereby, rendering noninfectious rotavirus particles (Ruiz et al., 1996). Once the outer layer is released, the noninfectious, double-layered viral particles are stable and presumably persist a long time in the marine environment (Loisy et al., 2004).

All the GSLs sampled were apparently clinically healthy, and we did not observe any symptoms of diarrhea. Further studies will be needed to determine whether rotavirus infections of GSLs are symptomatic and whether they affect their health status.

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