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Source: Journal of Wildlife Diseases, 34(1) : 39-46

Published By: Wildlife Disease Association

URL: <https://doi.org/10.7589/0090-3558-34.1.39>

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## VIRAL SEROLOGIC SURVEY OF BOWHEAD WHALES IN ALASKA

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**ABSTRACT:** Serum samples from 21 of 36 Eskimo harvested bowhead whales (*Balaena mysticetus*) were positive by virus neutralization (50% endpoint titer  $\geq 1:28$  and/or 100% endpoint titer  $\geq 1:20$ ) for antibodies to at least one virus serotype from the calicivirus family, vesicular exanthema of swine virus (VESV) and San Miguel sea lion virus (SMSV). Many animals were positive to more than one serotype when using the Spearman-Kärber (S-K) method for calculating antibody titers. The most common serotype detected was VESV F55 with 6 of 36 (17%) by the Monto and Bryan (MB) titer calculation method, and 17 of 36 (47%) by the S-K titer calculation method. Vesicular exanthema of swine virus 1934B antibody was detected in 3 of 36 (8%) and 5 of 36 (14%) whales using the MB and S-K methods, respectively. Vesicular exanthema of swine virus J56 antibody was detected in 3 of 36 (8%) by the S-K method only. All whales  $< 8.5$  m (estimated yearlings,  $n = 6$ ) were seronegative for VESV J56 and 1934B while 10% and 17% of the whales  $> 8.5$  m were positive, respectively. Whales assumed to be sexually mature ( $> 13$  m) had a higher prevalence of antibody to VESV 1934B and SMSV 8 than those  $< 13$  m. Gender had an effect on seroprevalence of antibody to VESV 1934B as titers  $\geq 1:28$  (S-K method) occurred in 18% of the females and 7% of the males. Antibody to other serotypes (SMSV 8 and 12) occurred less frequently ( $< 6\%$ ) at an antibody titer  $\geq 1:28$  by the S-K method. All 36 whale sera were negative for antibody to VESV-A48, B51, C52, D53, E54, G55, H54, I55, and K54; Tillamook calicivirus, and dolphin morbillivirus; and SMSV-1, 2, 4, 5, 6, 7, 9, 10, 11, and 13 by the S-K method.

**Key words:** Bowhead whale, *Balaena mysticetus*, calicivirus, morbillivirus, serology, survey.

### INTRODUCTION

The bowhead whale (*Balaena mysticetus*) numbers were greatly reduced through commercial whaling (Shelden and Rugh, 1996) and the largest remaining stock is estimated to number 8,200 (7,200 to 9,400, 95% confidence interval), have an annual rate of increase of 3.1% (1.4% to 4.7%, 95% confidence interval) (Zeh et al., 1995), and utilizes the Bering, Chukchi, and Beaufort Seas (Moore and Reeves, 1993; Shelden and Rugh, 1996). There is increasing evidence that some of these whales do not migrate extensively but rather remain in the Bering and Chukchi Seas throughout the year (Bogoslovskaya, 1982; Ainana et al., 1995; Zelensky et al., 1995). Those that migrate northward in the spring along the Chukchi Sea coast of Alaska and westward in the fall along the Beaufort coast of Alaska are subject to hunting by 10 coastal villages associated with the Alaska Eskimo Whaling Commission (AEWC). This subsistence hunt of cultural importance to the Alaskan Eskimo continues under careful regulation by the

International Whaling Commission (Braham, 1984) and specimen materials can be obtained from harvested whales (Albert, 1988; Stoker and Krupnik, 1993).

Examination of harvested whales at selected locations over the years has revealed only modest evidence of disease or injury other than that due to subsistence hunting. Reported injuries include propeller strikes (George et al., 1994), rope or net entanglements (Philo et al., 1992), intestinal volvulus (Heidel and Albert, 1994), fractured mandible (Philo et al., 1990), killer whale (*Orcinus orca*) bite (George et al., 1994), and skin penetration by Pacific walrus (*Odobenus romarus divergens*) tusk (Philo et al., 1993). Infectious diseases are very poorly understood as subsistence harvested whales are typically "healthy" and only isolated cases of ulcerated skin (necrotic epidermatitis) and a *Clostridium perfringens* and a *Fusobacterium* sp. associated abscess (jaw) have been reported (Philo et al., 1993). Potential pathogens have been isolated from bowhead whales but not associated with severe pathologic lesions or other impacts (Shotts et al. 1990; Philo et

al., 1993). Gastric nodules due to larval nematodes (Migaki et al., 1982) and male pseudohermaphroditism (Tarpley et al., 1995) has been reported. There is serologic evidence of exposure to several caliciviruses in four bowhead whales (Smith et al., 1986, 1987).

Viral (morbillivirus and calicivirus) diseases occur in many marine mammals including those cohabiting with bowhead whales and was a major reason for this survey. Morbillivirus epizootics occurred among harbor porpoises (*Phocoena phocoena*) (Kennedy et al., 1988), striped dolphins (*Stenella coeruleoalba*) (Domingo et al., 1990; Duignan et al., 1992) in Europe, and bottlenose dolphins (*Tursiops truncatus*) of the Gulf of Mexico (Lipscomb et al., 1996; Duignan et al., 1996). Caliciviruses have caused skin, lip, mouth, and tongue lesions (vesiculation and ulceration), abortion, encephalitis, and pneumonitis in various species (Smith et al., 1973, 1979, 1983; Barlough et al., 1986a). Marine caliciviruses are morphologically and physicochemically indistinguishable from vesicular exanthema of swine virus (VESV) and are capable of producing VESV-like disease in exposed pigs (Smith et al., 1973; Barlough et al., 1986a; Berry et al., 1990). The relevance of antibody to VESV-type viruses in bowhead whales and other marine mammals has little to do with swine (first described effected host), but it is related to the ecology of marine caliciviruses.

Serologic evidence of exposure to morbillivirus and calicivirus in many species has been documented world-wide and in regions frequented by bowhead whales. Morbillivirus antibodies have been detected in 68 of 191 polar bears (*Ursus maritimus*) from Alaska and Russia (Follman et al., 1996), and 92 of 100 long-finned pilot whales (*Globicephala melas*) and 16 of 25 short-finned pilot whales (*Globicephala macrorhynchus*) from the western Atlantic Ocean (Duignan et al., 1995). Marine caliciviruses have been isolated from, or antibody detected in, many marine mammals

including Steller sea lion (*Eumetopias jubatus*) from Alaska and Oregon (Barlough et al., 1987a; Skilling et al., 1987), California sea lion (*Zalophus californianus californianus*) (Barlough et al., 1987b), northern fur seals (*Callorhinus ursinus*), northern elephant seal (*Mirounga angustirostris*), Pacific walrus, and Atlantic bottlenose dolphin (Smith et al., 1981; Barlough et al., 1986a, 1986b; Berry et al., 1990). Tilmamook (bovine) calicivirus was not found in northern fur seals, Pacific walrus, seals (Phocidae), or several cetacean species (Barlough et al., 1987b).

A serologic survey for antibodies to viruses was conducted on bowhead whales sampled in 1980 indicating the presence of antibody to marine caliciviruses and VESV serotypes (Smith et al., 1987). In this study we evaluate sera from 36 bowhead whales sampled in 1993, 1994, and 1995 for calicivirus and morbillivirus serotypes. Large aquatic animals such as the bowhead whale, are difficult to observe over long periods and nearly impossible to manipulate and clinically examine while free-living. Therefore, serologic testing of harvested whales offers a valuable tool for disease monitoring. The major weakness for its use is that antibody titers can not assess the full meaning (infection, pathology, impact) of the exposure to these viruses. A better understanding of the temporal (year, season) and biological (age, sex) relationship of antibody prevalence to these viruses will provide insight into the ecology and relationships of these potential pathogens in the bowhead whale.

#### MATERIALS AND METHODS

Bowhead whales harvested in 1993–95 near Barrow, Alaska (USA) (71°17'N, 156°45'W) were from a region 2 to 32 km offshore bordered by longitudes 157°00'W and 155°30'W. Total length measurement of the whales was from the tip of the snout to the notch in the fluke. Serum samples were prepared from whole blood collected from the distal most portion of the hard palate mucosa by cutting the vascular rete. Collection was completed as soon as possible post mortem (usually 10 hr). Free flowing blood was collected in six to eight 10

ml red top (no additive) Vacutainers (Becton Dickinson, Rutherford, New Jersey, USA) and spun using a tabletop clinical centrifuge (IM-426 Centr-CL2 Centrifuge, International Equipment Company, Needham Heights, Massachusetts, USA) at 5,000 rpm. Serum was separated and stored frozen at  $-20^{\circ}\text{C}$ . Samples were then shipped frozen to the Foreign Animal Disease Diagnostic Laboratory (FADDL, Plum Island, New York, USA). Evidence of hemolysis, clot formation in the serum, or other unusual appearances were noted.

The serum samples were thawed, diluted 1:10 in sterile Eagles minimum essential salt media (EMEM, Biowhittaker, Walkersville, Maryland, USA) containing 25  $\mu\text{g}/\text{ml}$  gentamicin (Gensia Pharmaceuticals Inc., Irvine, California, USA) and 2.7  $\mu\text{g}/\text{ml}$  amphotericin B (Gensia Pharmaceuticals Inc.) and inactivated at  $56^{\circ}\text{C}$  for 30 min. A screening microtiter virus neutralization test for antibody to caliciviruses was performed in 96 well tissue culture plates (Costar, Cambridge, Massachusetts, USA) using 25  $\mu\text{l}$  serum diluted 1:10 in each of six wells. To three of the wells, 25  $\mu\text{l}$  virus suspension containing 100 tissue culture infectious doses-50% ( $\text{TCID}_{50}$ ) was added; the remaining three wells served as control to detect the serum's toxicity. The final dilution of serum in the well was 1:20 for the screening test.

The viruses tested were VESVs A48, B51, C52, D53, E54, F55, G55, H54, I55, J56, K54, 1934B; Tillamook; and SMSVs 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 13; all were from the FADDL repository. The SMSViruses and Tillamook calicivirus were provided by A. Smith. After 1 hr incubation at  $37^{\circ}\text{C}$  at 5%  $\text{CO}_2$  and 95% humidity, 100  $\mu\text{l}$  of Vero cells (for VESVs E54, F55, J56, and 1934B serotypes, IBRS-2 cells were used) suspension at 200,000 cells/ml EMEM containing 10% fetal bovine serum (National Veterinary Services Laboratory, Ames, Iowa, USA) were added. The plates were incubated under the same conditions described above for 3 days and examined microscopically for the presence of cytopathologic effect (CPE) in each well. If a sample was positive in the screening test for antibody to any virus, a titration was performed in which the sample was diluted two-fold and the test repeated with the dilutions and the selected virus. The serum titer was calculated by the Spearman-Kärber (S-K) method, giving 50% endpoints, and also by the Monto and Bryan (MB) method, giving 100% protection endpoints (Schmidt and Emmons, 1989; Monto and Bryan, 1974). For detection of antibody to the dolphin morbillivirus (provided by P. Duignan), the test protocol was identical except that 50  $\mu\text{l}$  of diluted serum was mixed with 50  $\mu\text{l}$

virus at 100  $\text{TCID}_{50}/50 \mu\text{l}$ , and the test was incubated seven days before reading. A serum was considered positive for antibody as calculated by the S-K method of determining 50% endpoints if the titer was  $\geq 1:28$ , and if the titer calculated by the MB method was  $\geq 1:20$ . All antibody titer calculations were based upon final serum dilutions. Mean titers are calculated from detected titers only; sera for which no titer was detected are not included in the calculation.

Statistical analyses included an ANOVA multifactor model with unequal sample sizes using the regression approach (Neter et al., 1985). The ANOVA model was used to compare serologic results and interactions of sex, season, and body length using SPSS® for Windows 6.0 (SPSS Inc., Chicago, Illinois, USA; Norusis, 1993). Correlation coefficients were determined using SPSS® for Windows 6.0 and only coefficients with  $P < 0.05$  are reported. Mean, standard deviation, and paired and unpaired  $t$ -test calculations were performed by Statview™ II for the Macintosh (1987 Abacus Concepts, Inc., Islandia, New York, USA).

## RESULTS

All sera sampled and tested were negative ( $<1:28$  for S-K method and  $<1:20$  for MB method) for antibody to VESVs A48, B51, C52, D53, E54, G55, H54, I55, K54, SMSVs 1, 2, 4, 5, 6, 7, 9, 10, 11, and 13, Tillamook calicivirus and dolphin morbillivirus. For VESV F55, using the MB method (100% protection) six whales had titers  $\geq 1:20$  (mean titer of 1:27) and were considered positive. For VESV F55, using the S-K method (50% protection) 17 (47%) whales had titers  $\geq 1:28$  (mean titer of 1:43) and were considered seropositive (Table 1). For VESV J56, no animals exhibited a titer when determined by the MB method. Using the S-K method, VESV J56 had three (8%) whales with titers  $\geq 1:28$  and were considered positive for antibody (Table 1). For VESV 1934B three whales had antibody titers  $\geq 1:20$  by the MB method (mean titer of 1:27) and using the S-K method five had titers  $\geq 1:28$  (mean titer of 1:36) and were considered seropositive (Table 1). Using the S-K method two animals had titers  $\geq 1:28$  for SMSV 8 (mean titer = 1:29) (Table 1). For

TABLE 1. The number (%) of reactors (titer  $\geq 1:28$  for S-K method) by sex, length, and season for vesicular exanthema swine virus (VESV) F55, J56, and 1934B, and San Miguel sea lion virus (SMSV) 8 and 12 in bowhead whales from Alaska.

Category	VESV F55	VESV J56	VESV 1934B	SMSV 8	SMSV 12
Female $n = 22$	10 (46)	2 (9)	4 (18)	1 (5)	1 (5)
Male $n = 14$	7 (50)	1 (7)	1 (7)	1 (7)	0
Spring $n = 23$	11 (48)	2 (9)	3 (13)	0	1 (4)
Fall $n = 13$	6 (46)	1 (8)	2 (15)	2 (15)	0
$\leq 8.5$ m <sup>a</sup> $n = 6$	2 (33)	0	0	1 (17)	0
$> 8.5$ m $n = 30$	15 (50)	3 (10)	5 (17)	1 (3)	1 (3)
$\leq 13$ m <sup>b</sup> $n = 28$	13 (46)	3 (11)	3 (11)	1 (4)	1 (4)
$> 13$ m $n = 8$	4 (50)	0	2 (25)	1 (13)	0
ALL $n = 36$	17 (47)	3 (8)	5 (14)	2 (6)	1 (3)

<sup>a</sup> 8.5 m = maximum length estimate for yearling (Koski et al., 1993).

<sup>b</sup> 13.0 m = length at sexual maturation (15–20 yr) (Koski et al., 1993).

SMSV 12, one animal exhibited a titer of 1:28 using the S-K method (Table 1).

Table 1 compares the percent positive reactors using the S-K method for the serotypes detected by sex, season, and length. Due to the very low sample size for animals sampled in 1994 we did not attempt to statistically evaluate year to year differences. More than double the percent females (18%) than males (7%) had significant antibody levels to VESV 1934B. Based on S-K method results only, gender had a significant effect ( $F = 3.21$ ,  $P = 0.08$ ) while length did not ( $F = 1.80$ ,  $P = 0.19$ ), although there appeared to be a higher incidence in whales  $> 13.0$  m (25%) than those  $\leq 13.0$  m (11%) (Table 1). The interaction of sex, length and season had the most effect ( $F = 4.52$ ,  $P = 0.04$ ) for VESV 1934B. Antibody to VESV F55 appears to be evenly distributed between the sexes (46% female and 50% male reactors), and for the different lengths;  $\leq 8.5$  m (33%),  $> 8.5$  m (50%), and  $> 13$  m (50%). The mean length (m) for the whales that were seropositive to VESV F55, J56, and 1934B are 11.1, 10.1, and 11.9, respectively, and not significantly different from each other ( $P > 0.05$ ). Two whales (15%) harvested in the fall were positive for SMSV 8 while no whales (0 of 23) in the spring were positive and season had a significant affect ( $F = 4.15$ ,  $P = 0.05$ ). There was a higher prev-

alence of antibodies to SMSV 8 for whales  $> 13$  m (13%) compared to whales  $\leq 13$  m (4%) (Table 1). As stated earlier, season had a significant effect, and the mean lengths for the whales from which serum was collected in the fall and spring (1993–95) were 12.3 and 10.4 m, respectively, and were significantly different ( $P = 0.017$ ). This difference in length by season may effect our analyses for VESV 1934B and SMSV 8.

Correlation coefficients were determined for all serotypes detected to determine potential cross-reactors and/or common exposures. The only significant correlation coefficient determined is  $-0.358$  ( $P = 0.032$ ) for VESV 1934B and F55. All remaining serotype correlation coefficients had a  $P > 0.05$ .

## DISCUSSION

Two of the caliciviruses tested elicited noteworthy, possibly significant, responses as calculated by the S-K method over the 3 yr studied: VESV F55 exhibited a prevalence of 47% and mean titer of 1:43; and VESV 1934B had a prevalence of 14% and mean titer of 1:36. Calculation of titers by the S-K method tends to produce higher titers compared to the MB method (Monto and Bryan, 1974). However, the trends are apparent with this second method as well: 17 and 8% of the samples exhibited titers to VESV F55 (mean titer of 1:27)

and VESV 1934B (mean titer of 1:27), respectively. All 36 serum samples were negative (antibody titers <1:28 by S-K method) for antibody to 20 of the 25 calicivirus serotypes tested and to the dolphin morbillivirus.

Interpreting serological test information from a species must be considered arbitrary when the antibody response of experimentally or known infected animals is not available. Young harp seals (*Phoca groenlandica*) inoculated with cell culture adapted SMSV 2 showed very small lesions and a meager virus neutralizing response (maximum titer 1:50) 10 days after inoculation. In swine inoculated with the same stock of virus, the virus neutralizing antibody titer was 1:300 at 10 days post inoculation (Gelberg, 1980). Generally, infection of domestic swine with swine virulent, swine adapted caliciviruses results in 50% endpoint neutralizing antibody titers greater than 1:500 (J. A. House, unpubl. data).

Evaluating antibody prevalence is further complicated by the methodology used to establish an antibody titer as positive. Over the years thresholds for animals serologically positive for calicivirus infections have varied. Positives have been considered when a serum sample shows full neutralization at  $\geq 1:10$  (Smith and Latham, 1978; Smith et al., 1987), full neutralization at  $\geq 1:20$  (Barlough et al., 1986a, b, 1987a, b, 1988; Berry et al., 1990), or 50% neutralization at  $\geq 1:10$  (Smith et al., 1978). The calculations used in all these studies were based upon a test for rhinovirus antibody (Monto and Bryan, 1974). Interpretations were apparently based upon the actual starting serum dilution of 1:10 (Smith and Latham, 1978; Smith et al., 1987) or perhaps a final serum dilution of 1:20 (Barlough et al., 1986a, b, 1987a, b, 1988; Berry et al., 1990). Standard procedures used in our study consider the final serum dilution in the well as the basis for calculation of antibody titers (e.g., a starting dilution of 1:10 represents a final serum dilution of 1:20 after the addition

of the test virus to the serum). If these assumptions on the performance and calculations of the 100% protection test are correct, the S-K method using a starting serum dilution 1:10 (final dilution 1:20) would be comparable to using a starting dilution of 1:10 or a final dilution of 1:20 for the 100% protection test. The 50% endpoint titers determined would then be higher. For example, a positive at a starting dilution of 1:10 with the 100% protection test would have a 50% endpoint titer of 1:28. For this reason we present two methods of titer calculation, and different titers for each method for determining if a serum sample is positive.

Length (presumably correlated with age) in combination with sex and season seems to have an effect on the seroprevalence of some calicivirus serotypes (VESV J56 and 1934B). Bowhead whales  $\leq 8.5$  m with short baleen ( $<1$  m) are likely to be yearlings (Koski et al., 1993). We chose to compare this small sized whale to larger whales so as to compare very young animals (likely  $\leq 1$  yr) to older ( $>1$  yr). The six whales that are  $\leq 8.5$  m had no antibodies to VESV J56 and 1934B while larger and older whales had higher prevalence rates. Antibody to only two viruses (VESV F55 and SMSV 8) was detected in whales  $<8.5$  m (probably yearlings or younger), and only VESV F55 occurred in more than one of the six young whales. This could be expected as young whales may have missed a past wave of viral exposure (VESV J56 and 1934B), but were exposed to a more recent viral wave (VESV F55). This information is very important in determining the periodicity of these viral serotypes in cetacean species. However, this may have resulted from a spatial effect since yearlings may not have been exposed to, or encountered, an area of higher exposure for the rest of the stock. Thirteen meters was selected as the length of sexual maturation (at approximately 15 yr) (Koski et al., 1993) for comparing seroprevalence in mature versus immature animals. For VESV 1934B there was a higher preva-

lence in longer whales than those  $\leq 13.0$  m. Gender had an effect on seroprevalence of antibody to VESV 1934B as positives occurred in more females than males, but the interaction of season and length was significant based on ANOVA, as well. It also was shown that larger whales are harvested in the fall, complicating the comparison of season or size independent of each other. An increase in prevalence for antibodies to SMSV 8 for whales  $> 13.0$  m was evident as well.

The absence of antibody to morbillivirus in all the whales tested is not surprising, as only 4 seropositive cetaceans have been found in the Pacific Ocean to date, and all have been dolphins in southern California (C. House, unpubl. data). However, antibodies to morbillivirus have been detected in polar bears in Alaska and Russia (Follman et al., 1996) and 41% of ringed seals (*Phoca hispida*) of arctic Canada (Duignan et al., 1977). Indeed, the spread of these viruses to the northern regions could be devastating as infections have been correlated to mass mortalities and pathological changes in other marine mammal populations (Duignan et al., 1995; Lipscomb et al., 1996). Serologic monitoring should continue in the bowhead whale as well as other arctic marine mammals of Alaska.

Evidence of calicivirus in bowhead whales, without virus isolation as the absolute test, is supported as outlined by Philo et al. (1993) in that (1) other marine mammals are known to be calicivirus reservoirs and found in the Bering Sea, (2) the presence of calicivirus antibodies in bowhead whales, and (3) the ability of calicivirus to infect bowhead whale lung cells in tissue culture. Bowhead whales and other wildlife could experience unknown adverse health effects (mortality and impaired reproduction) since we know these viruses have displayed interspecific transmission with fairly dramatic lesions. Regardless of the extent of viral replication in the bowhead whales, the notable prevalence of antibody to VESV serotypes 1934B and F55 implies that these viruses

or similar antigens are circulating in the marine environment about 60 and 40 yr, respectively, after their first identification in swine.

The impact of these viruses to the bowhead whale population can not be determined from this study. Smith et al. (1987) examined serum samples from four bowhead whales taken in the 1980 subsistence hunt. The samples exhibited low titers as calculated by the MB method to SMSV 5, SMSV 8, SMSV 10, substatinal titers to VESV J56 and K54 (reported as K56), and no titers to VESV F55 and 1934B (reported as VESV-1-34). No clear pattern of serotype detection can be established between these two studies possibly due to the small sample size or the length of time (13 yr) between sampling. It is possible that various caliciviruses circulate in different cycles (temporally and spatially) and serology reflects the current transient types. Many aspects of the bowhead whale and caliciviruses are still unanswered, particularly if the level of antibody reflects true infection and viral replication within the bowhead whale. If so, the obvious question is whether or not the bowhead whale experiences pathological changes due to infection? The fact that there are no clear patterns of antibody prevalence or titers makes these questions more academic than critical to the health of the bowhead whale population at this time.

#### ACKNOWLEDGMENTS

The authors are very grateful for the cooperation of Eskimo hunters in Barrow and for allowing us to sample these whales. Many individuals participated in the collection of blood samples and other data associated with the whales including R. Linnehan, P. Nader, M. Philo, T. Hepa, C. Orpin, A. Werth, J. Defranco, L. Quakenbush, D. Ramey, Z. Qian, S. MacLean, B. Akootchook, M. Wesselman, M. Johnson, C. Brower, P. Becker, J. Fadely, F. Nicklin, P. Barnes, A. Ingling, H. Brower, Jr., C. Clark, J. Burgener, K. Burek, and H. Huntington. Thanks are extended to A. Smith and P. Duignan for contributing viruses used in these assays. The authors thank T. Albert for reviewing this manuscript and for his administrative support.

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Received for publication 11 March 1997.