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Vesivirus Viremia and Seroprevalence in Humans

Alvin W. Smith,[1]* Patrick L. Iversen,[2] Douglas E. Skilling,[1] David A. Stein,[2] Karin Bok,[3] and David O. Matson[3]

[1]*Department of Biomedical Sciences, College of Veterinary Medicine, Laboratory for Calicivirus Studies, Oregon State University, Corvallis, Oregon*
[2]*AVI Biopharma, Inc., Corvallis, Oregon*
[3]*Center for Pediatric Research, Eastern Virginia Medical School, Norfolk, Virginia*

Pathogenic caliciviruses of the genus *Vesivirus* circulate in oceanic ecosystems and spread to and among terrestrial mammals. Isolation of *Vesivirus* from natural and laboratory infections in humans led to this investigation of *Vesivirus* seroprevalence and viremia. Sera from four groups were tested for antibodies to *Vesivirus* as follows: blood donors whose units were cleared for donation, blood donors whose units were not accepted for donation solely because of elevated blood liver alanine aminotransferase (ALT) concentrations, patients with clinical hepatitis of unknown but suspected infectious cause, and patients with clinical hepatitis of unknown cause but associated with blood transfusion or dialysis. Additionally, sera were tested for *Vesivirus* genome by three methods: dot-blot and two reverse transcription-polymerase chain reaction (RT-PCR) methods. The calculated seroprevalence against *Vesivirus* virions within these groups ($N = 765$) was 12%, 21%, 29%, and 47%, respectively ($P < 0.001$ for group differences). Additionally, 11 (9.8%) of 112 sera tested yielded RT-PCR amplicons that by nucleotide sequence were distinct from each other and related to known *Vesivirus*. These data indicate that some blood donors in the population tested have serologic evidence of previous *Vesivirus* infection and some also have *Vesivirus* viremia. These results justify further investigation of an association between *Vesivirus* infection and illness in humans. ***J. Med. Virol. 78:693–701, 2006.***

KEY WORDS: calicivirus; *Vesivirus*; human; seroprevalence; viremia

INTRODUCTION

Recent spread of newly recognized human pathogens from animal reservoirs, for example, Nipah virus, coronavirus associated with severe acute respiratory syndrome (SARS), and avian influenza strains, has focused renewed study of viral ecology [Enserink, 2000, 2003; Smith, 2000; Trampuz et al., 2004]. A relatively under-explored potential reservoir of newly emergent human pathogens is the marine environment [Smith et al., 1998b; Culley et al., 2003]. We previously described laboratory and natural marine *Vesivirus* calicivirus infections in humans [Smith et al., 1998a].

Vesivirus is one of four genera in the *Caliciviridae*, the others being *Norovirus* and *Sapovirus*, common causes of gastroenteritis in humans, and *Lagovirus*, widespread in Eurasia in rabbits and hares [Green et al., 2000]. Within the *Vesivirus* genus, serotypes of marine origin form a polyphyletic grouping distinct from those designated feline caliciviruses (FCVs) [Berke et al., 1997]. *Vesivirus* cycles naturally in oceanic reservoirs and a single *Vesivirus* serotype has infected species as diverse as fish, seals, shellfish, swine, cattle, and primates, including humans, and caused disease, sometimes severe, in marine and domesticated food-producing animals and humans [Smith et al., 1998a,b]. Classical virologic investigations established that epidemics of vesicular exanthema of swine virus (VESV) in North America from 1932 through 1959 were caused by serotypes of marine origin [Smith et al., 1978, 1980b; Smith, 2000]. The VESV epidemic serotypes ($N = 13$) are closely related to serotypes ($N > 25$) of San Miguel sea lion viruses (SMSVs) also of ocean origin and the few other *Vesivirus* serotypes ($N = 5$) isolated from terrestrial animals since 1970 [Smith et al., 1978, 1980b; Smith and Boyt, 1990; Neill et al., 1995, 1998; Matson et al., 1996; Reid et al., 1999; Smith, 2000]. Viruses in the marine environment other than caliciviruses also are

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*Correspondence to: Dr. Alvin W. Smith, 105 Magruder Hall, College of Veterinary Medicine, Oregon State University, Corvallis, OR 97331. E-mail: calicit@qwest.net

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being discovered, a recent example being the characterization of four possibly new virus families related to caliciviruses at a high taxonomic level [Culley et al., 2003; Lang et al., 2004]. Of interest, the VESV epidemics were controlled, in part, by cooking of raw garbage containing fish and pork scraps fed to swine [Smith et al., 1978, 1980b; Smith and Boyt, 1990].

The FCVs also are in the genus *Vesivirus* and neutralizing antibodies to them occur in marine mammals [Berke et al., 1997; Smith, 2000]. Other *Vesivirus* strains (N = 5) as yet only isolated from terrestrial sources have antigenic and/or genetic properties similar to the *Vesivirus* serotypes often referred as the marine caliciviruses (e.g., [Seal et al., 1995; Barlough et al., 1998; Smith, 2006].

Vesivirus infection can have a multiplicity of clinical outcomes, dependent upon the host and strain, which include encephalitis, hepatitis, myocarditis, pneumonia, spontaneous abortion, and dermatitis (reviewed in [Smith et al., 1998b, 2002a,b; Smith, 2000]). The human cases of marine *Vesivirus* disease which were described previously occurred in association with the North Pacific Basin, one in a laboratory scientist working with a marine *Vesivirus* who experienced a disseminated vesicular exanthem and the second in a field biologist working with marine mammals who had a vesicular exanthem on the face [Smith et al., 1998a]. One strain recovered from these human cases was by genome sequence related most closely to SMSV-5, the serotype known to infect more than 20 animal species and cause different clinical manifestations in them [Smith et al., 1977, 1980a, 1998b; Barlough et al., 1986]. The other strain from a human case was of a new *Vesivirus* serotype. To assess whether *Vesivirus* infections are more widespread among humans, the seroprevalence against *Vesivirus* and possible *Vesivirus* viremia were examined in adults whose donated blood was tested at a northwest-U.S. regional blood laboratory and, because of initial results, in patients with clinical hepatitis of unknown etiology.

MATERIALS AND METHODS

Serum Samples

Sequential, de-identified sera from blood donors were collected over a 4-year period (1996–1999), based upon availability, from the Regional Red Cross Blood Testing Laboratory in Portland, Oregon, which serves eight Western States and was processing samples from about 3,000 units of blood per day. One sample set included blood cleared for donation after clinical and laboratory screening, which included negative test results for hepatitis B surface and core antigens, HIV-1 and 2, HIV P24 antigen, human T-cell lymphotropic virus type 2, hepatitis C antigen, and serum alanine aminotransferase (ALT) levels below 120 International Units (IU). A second, concurrently acquired set of sequential blood specimens was collected from donors whose pre-donation laboratory values differed from the first set only in that the ALT level was $\geq$120 IU. Based upon testing of these two sets of samples, three additional sets of sera from cases of clinical hepatitis were acquired from an industry source. Two of these sets represented hepatitis cases of unknown but suspected infectious cause. Both were negative for known hepatitis viruses, but differed from each other in that some cases were associated with transfusion or dialysis. Other samples came from hepatitis cases caused by hepatitis B or C virus. The study was approved by the Institutional Review Boards of the authors' institutions.

Antigen Preparation

Antigen for serum antibody detection was generated from four genus *Vesivirus* family *Caliciviridae* serotypes, three SMSVs and one a feline *Vesivirus* prototype, as follows: SMSV-5, originally recovered from blisters on the flippers of a Northern Fur Seal in the Bering Sea and subsequently from skin lesions on a researcher [Smith et al., 1998a]; SMSV-13, recovered from large skin erosions on the flippers and mouth of a California Sea Lion (*Zalophus californianus)* at a marine mammal rescue facility and subsequently shown to cause similar disease in swine and cattle [Berry et al., 1990]; SMSV-17, initially isolated from infected shellfish and an aborted California Sea Lion fetus on the Santa Barbara Channel Islands off the coast of California and subsequently demonstrated to establish persistent infections in experimentally exposed shellfish [Smith, 2000; Burkhardt et al., 2002]; and prototype FCV F9 vaccine strain [Meanger et al., 1992].

Each virus strain was grown in roller bottles to a titer of $\sim 10^7$ plaque-forming units per ml in Vero cells. When cytopathology was scored 4+ ($\sim$24 h post-inoculation), cells were lysed by freeze-thawing, cellular debris was removed by centrifugation and the supernatant applied to a continuous CsC1 gradient with a mean density of 1.35 g/ml. Virus bands were visible at a density of about 1.36 g/ml and were removed, cleared of CsC1 by dialysis and checked for purity and concentration by direct electron microscopic examination. Optimal dilutions of individual purified virus stocks were determined, then equal parts of each of each SMSV stock were pooled and both preparations (SMSV pool and F9) were retitrated for optimal dilution.

Enzyme Immunoassay (EIA) for Antibody Detection

The EIA utilizing the SMSV pool antigen was described previously [Smith et al., 1998a]. SMSV pool antigen was applied directly to the plate and then incubated with a 1:100 dilution of human serum. Bound immunoglobulin was detected by an alkaline phosphatase-conjugated, anti-human immunoglobulin second antibody (1:40,000 dilution; Sigma, St. Louis, MO). Optical densities (ODs) of the color substrate Blue Phos (KPL, Gaithersburg, MD) were read at 650 nm on a Titertek Multiskan Plus EIA Plate Reader (Titertek, Huntsville, AL). For comparison, sera were tested against wells with no viral antigen. EIA data were

analyzed as P-N values, in which the OD of the antigen-negative well (N) was subtracted from the OD of the well containing viral antigen (P). The OD values of antigen-negative (N) wells were $\leq$0.005 and most were 0.000. The cut-point for positivity was a P-N $\geq$0.043. Previous testing [Smith et al., 1998a] demonstrated antibody in EIA-positive samples by Western blot and by preabsorption with SMSV-5 antigen, and that paired sera from cases of Norwalk virus gastroenteritis showed no seroresponse to the SMSV pool. *Vesivirus* typing antisera show broad cross-reactivity of the SMSV-5 capsid antigen with that of other *Vesivirus* serotypes [Kurth et al., 2006].

The FCV antigen also was utilized in the same EIA, in which all other reagents and steps were the same. The cut-point for the FCV antigen also was a P-N value $\geq$0.043, which was derived by utilizing antigen of assay potency comparable to that of the SMSV pool and a graphical method for estimating cut-point [Matson, 1999].

Vesivirus Nucleic Acid Detection and Characterization

To test the possibility that sera may contain *Vesivirus* genomes, as suggested by the clinical evidence of viremia in cases of *Vesivirus* illness, including humans [Smith et al., 1998a], three complementary methods targeting three *Vesivirus* genomic regions were utilized to detect *Vesivirus* RNA in serum (Fig. 1): *dot blot* for the ORF1 3C protease region, *reverse transcription-polymerase chain reaction (RT-PCR)* for the 3′ terminal region of ORF1 encoding a portion of the viral RNA polymerase or for a portion of the viral capsid protein, and *nucleotide sequencing* of RT-PCR amplicons.

For the *dot-blot method*, a 280-bp biotin-labeled riboprobe (5R.3) specific for the *Vesivirus* 3C protease genomic region (designed from SMSV-5 and corresponding to nts 3,727–4,007 of primate *Vesivirus* prototype *Pan-1*, GenBank number AF091736) was synthesized by in vitro transcription from recombinant plasmid pCN5R.3 (not shown) and probed against 50 ng of total RNA extracted from blood donor serum using Trizol (Invitrogen, Inc., Carlsbad, CA) blotted onto a nylon membrane. Comparison controls also blotted included 20 ng of RNA extracted from CsCl-banded SMSV-5 and 50 ng of total RNA from mouse embryo (Clontech, Inc., Palo Alto, CA). The membrane was probed at 50°C in

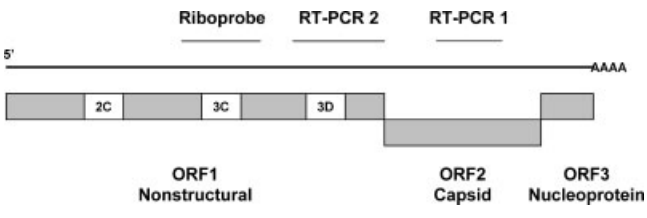

Fig. 1. Genome organization of *Vesivirus* and genome regions detected in this study. The *Vesivirus* genome detection and characterization were directed at two regions of ORF1 and one region of ORF 2 with complementary methods (drawing not to scale).

Northern Max Hyb Buffer (Ambion, Inc., Austin, TX) and washed at high stringency (2X SSC, 1% SDS, 60°C).

For one *RT-PCR* (RT-PCR 1), total RNA was extracted from 100 µl of serum using Trizol. A positive control (tissue culture of primate calicivirus) and a negative control (water) were included in each round of extraction in order to control for the quality of the extraction. Extracted RNA was diluted in water containing 0.4 U/µl of RNAsin (Promega, Madison, WI) and used directly in RT-PCR assays. The RNA was amplified with primers D3A 10F (5′-CCAAA GCCAA CAACC GTTGG TTCCA TG-3′, designed from prototype A48 strain, GenBank number U76884, and corresponding to nts 6,962–6,988 of *Pan-1*) and D3A 481R (5′-GTGTA GCAAT CCTGA CAACT TTGCT GG-3′, designed from prototype A48 strain, and corresponding to nts 7,415–7,441 of *Pan-1*) and yielding a product of approximately 480 nt. This RT-PCR utilized Enhanced Avian Reverse Transcriptase (Sigma) according to manufacturer's instructions and Platinum Pfx DNA Polymerase (Invitrogen) also following the manufacturer's recommended protocol. For the second RT-PCR (RT-PCR 2), total RNA from serum was amplified in a similar manner using primers 289rc, 5′-TATGG TGATG ACGGG GTCTA CA-3′ (designed from nucleotides 5,123–5,144 of *Pan-1*), and primer "pre-capsid," 5′-CACCT CACCA CTGAG CCC-3′ (designed from nucleotides 5,650–5,633 of *Pan-1*), and yielding a product of approximately 510 base pairs. RT-PCR amplicons were resolved in a 1% agarose gel and visualized by ethidium bromide under UV light.

For *nucleotide sequencing* of RT-PCR amplicons, RT-PCR products were sequenced directly or after cloning. Cloning used the pGEM-T vector system I (Promega) following the manufacturer's protocol. Recombinants obtained from cloning were screened by PCR using forward and reverse primers targeting the SP6 and T7 polymerase promoters bracketing the multiple cloning cassette. Plasmid DNA from the positive clones was purified by QIA prep Spin Miniprep Kit (Qiagen, Valencia, CA) and sequenced with Sequi Therm EXCEL II Long-read DNA Sequencing Kit (Epicentre Technologies, Madison, WI) on an automated sequencer (ALF Express DNA Sequencer, Amersham Pharmacia, Piscataway, NJ).

Sequence Comparisons

Raw DNA sequences were edited in OMIGA 2.0 (Accelrys, San Diego, CA). Sequences were compared and aligned in CLUSTAL W with prototype sequences retrieved from the NCBI public databases using BLAST searches. Comparison *Vesivirus* sequences included human *Hom-1* (GenBank number U623227), primate *Pan-1* (AF091736), SMSV-2, SMSV-5, SMSV-6, SMSV-7, SMSV-13, and SMSV-14 (U18730, U18731, U18732, U18733, U18734, and U18735), VESV strains A48, C52, E54, I55, and 1934b (U76874, U18738, U18739, U18740, and U18736), bovine (U18741), feline strain F9 (M86379), rabbit (AJ866991), skunk strains 4-1L, 4-2S, and 7-2 (U14668, U14670, and U14672), and

walrus (AF321298). Two sequences of SMSV-1 (U15301 and U14676), called here SMSV-1A and SMSV-1B, and of SMSV-4 (U15302 and U14674), called here SMSV-4A and SMSV-4B, were included.

GenBank numbers of sequences utilized or determined in this study include: for the 3C region riboprobe, DQ300285; for the capsid region amplicon of RT-PCR 1: sample 4, DQ300286; for the polymerase region amplicons of RT-PCR 2: sample N102, DQ300287; sample N104, DQ300288; sample 214, DQ300289; sample 298, DQ300290; sample 310, DQ300291; and sample N330, DQ300292.

Definitions

Healthy blood donors (Normal) are adults volunteering to donate blood and whose donation cleared screening tests and was accepted for donation. Most are repeat donors. Persons with elevated ALT (high ALT) are other blood donors, like the healthy donors, except their blood donation was rejected solely because of the detection of elevated serum ALT values in the blood at the time of donation. Samples from persons with infectious hepatitis (B and C) and hepatitis of unknown etiology (non-A-G hepatitis) came from an industry repository established to discover new causes of human hepatitis.

Statistical Analysis

Optical density (P-N) values were compared between groups by ANOVA and, for correlation, by regression. Difference in estimated prevalence of anti-*Vesivirus* antibodies among groups were assessed by the χ^2 test. The trend of estimated prevalence among groups with increasingly greater evidence of hepatitis was assessed by the Mantel χ^2 for the trend. *P*-values <0.05 were considered significant.

RESULTS

Prevalence of *Vesivirus* Antibody in Different Groups

A total of 374 sera from Normal and 350 sera from high ALT blood donors were tested against both *Vesivirus* antigens. Results from this testing led to obtaining a set of sera from patients with hepatitis (see Materials and Methods) and retesting against the SMSV pool antigen because the estimated prevalence of antibody to both antigens in the high ALT donors was about twice that observed among the Normal blood donors ($P < 0.05$, Yates' corrected χ^2 for each comparison) and mean P-N values were significantly higher among the high ALT donors than among the Normal donors when tested against the SMSV pool antigen but not when tested against the F9 antigen ($P < 0.05$, Student's *t*-test). The sera from all groups were recoded and tested together; the results of this combined testing are presented in Table I.

In the retesting, high ALT donors had a higher prevalence of antibody to both test antigens (21% for the SMSV pool and 14% for F9) than the Normal donors ($P < 0.001$ and $P = 0.026$, respectively). The retesting also yielded a higher mean P-N value among positive samples in the high ALT donors for antibody to the SMSV pool ($P < 0.001$), but not for antibody to F9. Sera from 51 cases of clinical hepatitis had a yet higher estimated prevalence (25%) of anti-SMSV pool antibody

TABLE I. Anti-*Vesivirus* Serum Antibody Among Different Study Groups

	SMSV antigen		Feline CV antigen	
Group[a]	No. positive/no. tested[b]	Mean (SD) P-N of Pos. samples[b]	No. positive/No. tested#	Mean (SD) P-N of Pos. samples
Normal	44/374 (12)[c,d,e,f,l]	145 (243)[h,i,j]	31/374 (8.2)[k]	214 (320)
High ALT	73/350 (21)[c,f,l]	241 (303)[h]	48/350 (14)[k]	192 (250)
Hepatitis cases	13/51 (25)[d]	267 (408)[i]	ND	ND
Non-A-G hepatitis	12/41 (29)[e]	278 (425)[j]	ND	ND
Associated with transfusion or dialysis	7/15 (47)[f,g,l]	102 (65)	ND	ND
Exposures source unknown	5/26 (19)	524 (560)	ND	ND
HBV or HCV hepatitis	1/10 (10)	139 (—)	ND	ND

[a]"Normal" = blood donors cleared for donation by all screening procedures. "Elevated ALT" = blood donors whose units were rejected only because of elevated liver ALT; "Non-A-G hepatitis" = cases of clinical hepatitis suspected to be of infectious etiology, but not linked to any known etiologic agent of hepatitis.
[b]Statistically significant comparisons between groups are indicated by lower-case letters, as follows.
[c]$P = 0.001$.
[d]$P = 0.013$.
[e]$P = 0.004$.
[f]$P < 0.001$.
[g]$P = 0.041$.
[h]$P < 0.001$.
[i]$P = 0.022$.
[j]$P = 0.034$.
[k]$P = 0.026$.
[l]$P < 0.001$.
Tests of proportions between groups by Yates' corrected χ^2 or Mantel χ^2 for the trend. Tests of means of samples reacting positively by Student's *t*-test when variances were normally distributed or by the Kruskal–Wallis H test when significant differences between variances occurred [Dean et al., 1994].

and mean P-N value among positive samples. When these hepatitis cases were analyzed by subgroup, the highest prevalence (47%) of antibody to the SMSV pool was in the cases of Non-A-E hepatitis associated with transfusion or dialysis. Cases of Non-A-E hepatitis with an unknown exposure source had an estimated prevalence (19%) similar to that (21%) of the high ALT donors and cases of HBV or HCV hepatitis had an estimated prevalence (10%) similar to that of healthy donors. The increase of anti-SMSV pool prevalence along the axis "Normal donor–high ALT donor–Non-A-E hepatitis case associated with transfusion or dialysis" was highly significant ($P < 0.001$, χ^2 for the trend). The mean P-N value of the positive samples from the Non-A-E hepatitis group was lower than that of the other groups studied ($P > 0.05$ for these comparisons). The patterns of significant differences in estimated prevalence were similar and statistically significant for a range of antibody assay cut-points, up to 0.200, although estimated prevalence declined as the cut-point increased.

Detection of *Vesivirus* RNA in Human Sera

Total RNA was extracted from 30 donor sera and tested by RT-PCR 1. The laboratory performing this testing had no prior experience with *Vesivirus* genomes in the facility. RT-PCR 1 generated a *Vesivirus* amplicon in one of these samples that was confirmed by dot-blotting (Fig. 2) and sequencing.

To reduce the possibility that a positive result for serum *Vesivirus* genome detection was generated by laboratory contamination at the first facility, a further 82 donor samples were tested in a second laboratory routinely performing RT-PCR and that had prior experience with *Vesivirus* RNA, but using newly designed primers to amplify a genomic region not previously amplified (RT-PCR 2). Ten of these 82 samples yielded amplicons of the expected size (Fig. 3).

Ten (11%) amplicons came from 91 high ALT donor samples and 1 (4.8%) amplicon from 21 Normal donor samples ($P = 0.64$, Yates' corrected χ^2). Five (15%) of the amplicons were from 34 sera that scored EIA-positive and 6 (7.7%) from 78 sera that scored EIA-negative ($P = 0.42$, Yates' corrected χ^2).

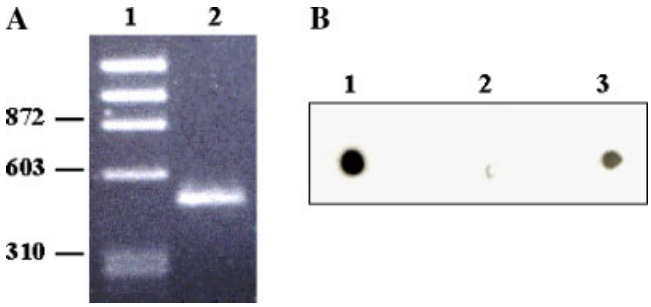

Fig. 2. RT-PCR 1 product and confirmatory dot blot. RT-PCR 1 testing of blood donor sera revealed an amplicon of the expected size for *Vesivirus* RNA (**A**) and dot blot probing of total RNA from the same serum provided evidence of *Vesivirus* viremia (**B**). In (A), **Lane 1**: molecular weight ladder; **lane 2**: RT-PCR 1 amplicon. In (B), dot 1 is 20 ng of RNA extracted from CsCl-banded SMSV-5, dot 2 is 50 ng of total RNA from mouse embryo, and dot 3 is 50 ng of total RNA extracted from the donor serum sample.

Sequence Comparisons of the RT-PCR Amplicons

Six amplicons from the RT-PCR 2 (polymerase region) sample set and the one from the RT-PCR 1 (capsid region) sample set were successfully sequenced. Amplicons not successfully sequenced yielded a band too faint for successful direct sequencing and were not successfully cloned. Five of the six polymerase region amplicons were distinct from each other but closely related (1–6% divergence). The sixth amplicon, study number N104, was distinct from the other five polymerase region amplicons (24–38% divergence from N104). When the five similar polymerase region amplicons were compared with GenBank entries spanning the amplification region of RT-PCR 2, the two best matches were with primate *Vesivirus Pan-1* (88–96% identity) and SMSV-6 (88–94%) and lower match was with SMSV-5 (84–86%).

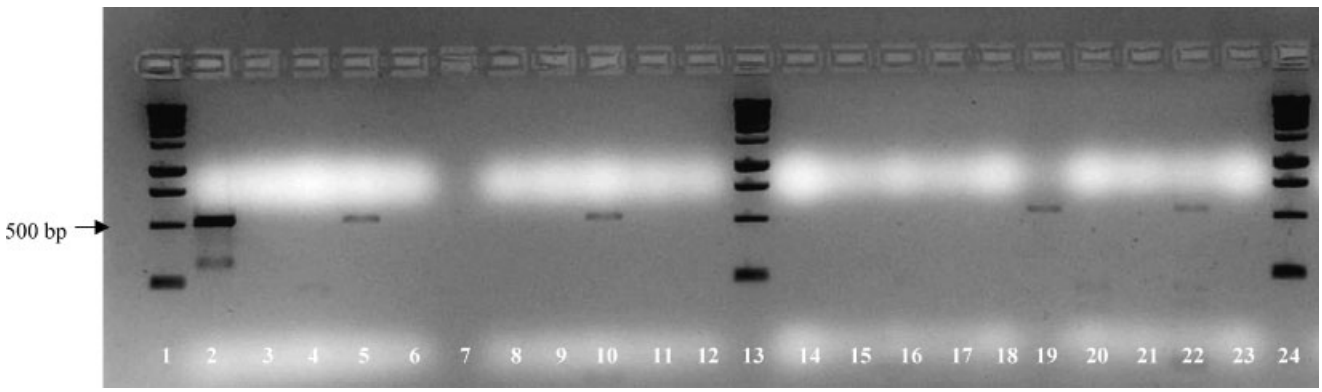

Fig. 3. RT-PCR 2 products from blood donor sera. Several amplicons generated by RT-PCR 2 were of the expected size (**lanes 5**, **10**, **19**, and **22**). **Lanes 1**, **13**, and **24**: molecular weight ladder. **Lane 2**: positive-control RT-PCR 2. **Lanes 3**, **11**, and **20** are negative controls containing all RT-PCR reagents without test specimen.

The sixth polymerase region amplicon (N104), was closest (68% identity) to SMSV-13 and 44% identical to F9. The capsid gene sequence from RT-PCR 1 best (97% identity) matched SMSV-5.

A smaller nucleotide (113 nt) region of the six polymerase region amplicons was shared with 22 SMSV-like *Vesivirus* entries in GenBank (Fig. 4). The five similar polymerase region amplicons were distinct from each of the GenBank entries and diverged from published *Vesivirus* sequences for the most part at sites where other *Vesivirus* strains also differed from each other (Part A). The amplicon from donor N104 also had a *Vesivirus* sequence, but was distinct from other *Vesivirus* strains, including F9, differing from published *Vesivirus* most at the 5′ end of the sequence, in a manner akin to how FCV differed from SMSV-like *Vesivirus* strains. Strain *Hom-1* derived from a skin vesicle of a previously reported case of human *Vesivirus* infection [Smith et al., 1998a] had a short (47–64 nt) region of overlap with the serum-derived polymerase region amplicons. In this region of overlap, nucleotide sequence identity with the polymerase region amplicons ranged from 66% to 83%.

DISCUSSION

In this report, the detection of antibodies to *Vesivirus* and of SMSV-like *Vesivirus* viremia in blood donors is described at a U.S. regional blood bank serving eight Northwestern states. A higher prevalence of anti-*Vesivirus* antibody was observed, in comparison with normal blood donors, in donors with elevated serum ALT values, and the highest prevalence, among the groups tested, in cases of clinical hepatitis of unknown etiology associated with transfusion or dialysis. In addition, *Vesivirus* RNA was detected in healthy and high ALT blood donors, occurring numerically but not statistically significantly more frequently in the high ALT blood donors than among the healthy donors tested. The findings of this study and the attributes of *Vesivirus* calicivirus strains in mammals extend the potential for *Vesivirus* disease in humans from a few well-described cases to a broader population.

The complementary evidence for viremia included positive results from separate laboratories testing separate sample sets, utilization of RT-PCR assays that amplified non-overlapping genomic regions, direct detection of genomic RNA by hybridization, and amplicon sequencing that revealed non-identity of sequence compared with known *Vesivirus*, including strains characterized in one of the laboratories previously [Rinehart-Kim et al., 1999] and among the set of characterized amplicons themselves. These results together indicate that the positive laboratory results were not because of contamination in sample collection and handling.

The antigens utilized for detection of anti-*Vesivirus* antibody were purified by cesium chloride banding, which yields a homogenous population of viable particles. A pool of three SMSV strains was utilized, with each strain representing a different potential mechanism for exposure of humans to *Vesivirus*: SMSV-5 detected previously in a human case, SMSV-13 known to cause disease in two livestock species, and SMSV-17 recovered from edible shellfish. The separate phylogenetic cluster within the *Vesivirus* genus defined by FCV was represented by a live vaccine strain widely administered to cats. The noted differences among serum groups in patterns of estimated prevalence remained across a broad range of potential assay cut-points. It would be unexpected for humans to have *Vesivirus* viremia, but not exhibit serologic evidence of infection.

Viremia was expected to be part of the natural history of *Vesivirus* infections in humans because one of two cases described had a disseminated vesicular exanthem of the hands and feet from which *Vesivirus* was cultivated [Smith et al., 1998a]. The *Vesivirus* strains causing *Vesivirus* viremia in this study were closest by genome sequence comparison to SMSV, marine *Vesivirus*. SMSV *Vesivirus* are widely distributed in marine and some terrestrial animals and may routinely "traffic" among these hosts, cycling from their large marine reservoirs onto land and perhaps back again. An ocean presence has been established by the isolation of virus, the presence of specific neutralizing antibodies, or by genome amplification and sequencing for 43 of the 46 known serotypes of the genus *Vesivirus*, including serotypes isolated initially from terrestrial hosts and named feline (FCV), primate (PCV *Pan-1*), bovine (BCV *Bos-1*), reptilian (RCV *Cro-1*), swine (VESV-A_{48}-K_{56}), mink (MCV), and human (SMSV-5 *Hom-1* and HuCV *Hom-1*) caliciviruses [Evermann et al., 1983; Smith et al., 1983, 1998a,b, 2002b; Seal et al., 1995; Reid et al., 1999]. Another marine *Vesivirus* isolated from walrus (WCV) causes hepatitis in domestic animals [Smith, 2000; Ganova-Raeva et al., 2004]. *Vesivirus* caliciviruses are resistant to environmental degradation; stable in aquatic substrates; multiply to high titer, with an estimated 10^{13} virions released into the ocean daily by a single California gray whale (*Eschricitus gibbosus*) [Smith et al., 1998b, 2004] and, in the case of FCV, have a cosmopolitan distribution [Studdert, 1978; Smith et al., 1998b]. Such attributes indicate the potential for frequent contact between a diversity of *Vesivirus* biotypes and hosts.

Known examples of such interaction include mussels and oysters, aquatic filter feeders that concentrate particulates, including viruses, from the water column, preserve viral viability for 60 days or more and thereby can deliver large doses of viable *Vesivirus* to species ingesting contaminated shellfish [Smith, 2000; Burkhardt et al., 2002]. Another example is from the mid-Pacific (French Frigate Shoals), where *Vesivirus*-infected fingerlings of two fish genera (*Aterinomorous spp.* and *Encrasicolina spp.*) were eaten by white tern (*Gygis alba*) hatchlings that developed a *Vesivirus*-associated blistering disease of the feet [Poet et al., 1996]. As mentioned above, whales (California gray) that can shed large numbers of *Vesivirus* particles per

A

Vesivirus Strain	Nucleotide Sequence
chimpanzee Pan-1	ACCACTCATA TCATCTGTCA TGCCCAAAGT CTTCACCAAC CTGAAACAGT TTGGTCTGAA ACCGACCCGG ACCGACAAAA CGGATGCTGA GATAACGCCT ATCCCTGCTG ATG
Study N330	---------- ---------- ---------- ---------- ---------- ----C----- ---------- ---------- ---------- ---------- ---------- ---
Study N102	---------- ---------- ---------- ---------- ---------- ----G----- ---------- ---------- ---------- ---------- ---------- ---
Study 310	--G---T--- ---------- ---------- ---------- ---------- ----C----- ---------- ---------- ---------- ---------- ---------- ---
Study 214	-G-------- ---------- ---------- ------G--- ---------- ----C----- ---------- ---------- ---------- ---------- ---------- ---
Study 298	---------- --G------- ------T--- ------G--- ---------- ----C----- ---------C -------G- ---------- ---------- ---------- --
SMSV-6	G--------C ---------- ---------- ----T----- ---------- ----C----- ---------- ---------- ---------- ---------- ---------- ---
VESV E54	---------C ---------- --A------- ----G-T--- --A------- ----C----- ---------- ---------- ---------- ---------- ------CAC- ---
VESV A48	G--G-----C ---------- ---------- ----G-G--- ----G----- ----C----- ---------- --------GT ---------- ---------- ---------- ---
SMSV-4A	---G-----C ---------- -------T-- ----G-A--- ---------- ----C----- ---------- ---------T ---------- ---------C ---------- ---
skunk 4-2S	---G-----C ---------- -------G-- ----G-A--- ---------- ----C----- ---------- ---------T ---------- ---------C ---------- ---
SMSV-4B	---G-----C ---------- -------G-- ----G-A--- ---------- ----C----- ---------- ---------T -------C-- ---------C ---------- ---
VESV 1934b	---C------ --C------- ---------- ------A--- --ACG----- ----C----- --------- ---------- ---------- ---T------ ---------- ---
skunk 4-1L	---G-----C ---------- ---------- ----T-A--- --ACG----- ----A----- ------T--- ---------- ---------- A--------C ---------- ---
skunk 7-2	---G-----C ---------- ---------- ----T-A--- --ACG----- ----A----- ------T--- ---------- ---------- A--------- ---------- ---
SMSV-1A	---G-----C ---------- ---------- ----T-A--- --ACG----- ----A----- ------T--- ---------- ---------- A--------- ---------- ---
SMSV-2	G--------C --C------- ---------- ----T-A--- T-ACG----- ----C----- G--------- ---------- ---------- ---------- ---------- ---
SMSV-1B	---G-----C ---------- ---------- ----G-G--- --ACG----- ----C----- ---------- ---------G ---------- ---------- ---------- ---
SMSV-5	---T-----C --T------- ---------- ---TG-G--- --ACG----- -C--C----- ---------- -----T---- ---------- ---------- --------A- ---
walrus	---------T -----C---- ----A----- ----G-G--- ---------- ----C----- ---------- ---------- ----C----- ---------A -----CAAA- ---
SMSV-7	---------T -----C---- ----G----- ---TG-G--- ---------- ----G----- ---------- ---------- ----C----- ---------- -----CAAA- ---
rabbit	---G-----C ---------- ----G----- ---TG-A--- --A------- ----C----- ---------- ---------- ----C----- ---------- -----CA-A- ---
VESV C52	---G-----C ---------- ----G----- ---TG-A--- --A------- ----C----- ---------- ---------- ----C----- ---------- -----CA-A- ---
bovine	---G-----C ---------- ----G----- ---TG-A--- --A------- ----C----- ---------- ---------- ----C----- ---------- -----CA-A- ---
VESV I55	---------C -----A---- --A------- ----G-T--- -----GAC-- ----C----- ---------- ---------- ---------- ---------C -----CCAC- ---
SMSV-14	---------C -----A---- ----G----- -C--G----- ---------- ----C--A-- G--------- ---------- ----C----- ---------A -----C---- ---
SMSV-13	---T-----C --T------- ------G--- ---------- ---------- ----C----- ---------- ---------- ---------- --------T- ---------- ---
Study N104	G--CGAGGCT CAT-G-CGGT C-G-G--G-- G-----G--G ---GG----- ----A---G- ---------- ---------- --A------- ---------- ---------- ---
feline F9	TATTA-GTAT G--AG-A-T- GTGA-C--A- T--TGGA--T --TTCTTCC- A---C----- ---A--T--- GTT-----GT ---T--GA-C A--TGA---- --TGA-C--- -CT

Fig. 4. Nucleotide (**A**) and amino acid (**B**) alignment of shared sequence among *Vesivirus* polymerase region amplicons from blood of study subjects and from *Vesivirus* strains represented in GenBank. Study strain sequences shared with these GenBank entries are aligned and placed next to the strains with which they had the highest sequence identity. GenBank sequences are grouped according to similarity to each other. Five of the study strains were similar to each other and closest to chimpanzee *Pan-1* and San Miguel sea lion serotype 6 strains. The other polymerase study strain also was closest to known *Vesivirus*, but in the pattern of sequence homology similar to feline calicivirus.

B

Vesivirus Strain	Amino Acid Sequence
chimpanzee *Pan-1*	PLISSVMPKV FTNLKQFGLK PTRTDKTDAE ITPIPAD
Study N330	---------- ---------- ---------- -------
Study N102	---------- ---------- ---------- -------
Study 310	**R**--------- ---------- ---------- -------
Study 214	**A**--------- ---------- ---------- -------
Study 298	--------**I**- ---------- ---------- -------
SMSV-6	---------- -S-------- ---------- -------
VESV E54	-------T-- -A-------- ---------- -----H-
VESV A48	---------- -A--R----- ------S--- -------
SMSV-4A	--------N- -A-------- ---------- -------
skunk 4-2S	---------- -A-------- ------S--- -------
SMSV-4B	---------- -A-------- ------S--- -------
VESV 1934b	---------- ----R----- ---------- -------
skunk 4-1L	---------- -S--R----- ---------- -------
skunk 7-2 & 3L	---------- -S--R----- ---------- -------
SMSV-1A	---------- -S--R----- ---------- -------
SMSV-2	---------- -S--R----- ---------- -------
SMSV-1B	---------- -A--R----- ------A--- -------
SMSV-5	---------- -A--R----- ---------- -------
walrus	---------- -A-------- ---------- -----K-
SMSV-7	---------- -A-------- ---------- -----K-
rabbit	---------- -A-------- ---------- -----T-
VESV C52	---------- -A-------- ---------- -----T-
bovine	---------- -A-------- ---------- -----T-
VESV I55	-------T-- -A---T---- ---------- -----H-
SMSV-14	---------- LA-------- ---------- -------
SMSV-13	--------R- ---------- ---------- --L----
Study N104	**-EAHCRSA-- --K-G----E -------N-- -------**
feline F9	IMYA-ISDQI -G--SSY--- ---V--SVGA -E--DP-

Fig. 4. (*Continued*)

gram of feces per day also migrate thousands of miles annually between the Sea of Cortez and the Arctic Ocean [Akers et al., 1974; Smith et al., 2004]. *Vesivirus* has been recovered at titers of 10^7 infectious virions per gram of spleen in naturally and experimentally infected opal-eye fish (*Girella nigricans*). These fish are resident along the Southern California coast [Smith et al., 1980a,b, 1981] and are a sports and commercial fish and a common food source for seals, some of which also have extensive migration cycle. Seals reproduce on land or ice, where *Vesivirus*-induced reproductive failure and death occur, where foraging scavengers can further redistribute the viruses into terrestrial ecosystems, and where exposure to *Vesivirus* likely occurred for one human case [Smith et al., 1998a].

The present findings indicate a broader potential for *Vesivirus* infection and, perhaps, illness in humans than previously recognized. The strains causing viremia and to which antibody was detected in this study are similar to the *Vesivirus* with an ocean reservoir. A finding of subclinical viremia and the detected highest seroprevalence in cases of clinical hepatitis associated with transfusion or dialysis suggest that blood exposure that may have led to hepatitis also could lead to higher exposure to *Vesivirus*. If *Vesivirus* causes hepatitis in humans, then an estimated rate for such causation can be derived from the study findings, as follows: the rate of 10 of 91 donors amplicon-positive in the serum who also had elevated serum liver transaminases (ALT) values, together with the rate of high ALT values occurring in about 1 in 1,000 blood donors, would correspond to a rate of ~1 in 10,000 blood donors who might have active, subclinical *Vesivirus* hepatitis. The association of higher anti-*Vesivirus* antibody prevalence with clinical hepatitis of unknown etiology would require further study. The finding of *Vesivirus* viremia in otherwise normal blood donors indicates that blood exposure to caliciviruses of the genus *Vesivirus* could occur by multiple routes of exposure. The diversity of host species, mechanisms of exposure and tissue tropisms for *Vesivirus* with the findings of this study suggest additional *Vesivirus* disease manifestations might be found in humans with further investigation.

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REFERENCES

Akers TG, Smith AW, Latham AB, Watkins HM. 1974. Calicivirus antibodies in California gray whales (Eschrichtius robustus) and Steller sea lions (Eumetopias jupatus). Arch Gesamte Virusforsch 46:175–177.

Barlough JE, Berry ES, Skilling DE, Smith AW, Fay FH. 1986. Antibodies to marine caliciviruses in the Pacific walrus (Odobenus rosmarus divergens Illiger). J Wildl Dis 22:165–168.

Barlough JE, Matson DO, Skilling DE, Berke T, Berry ES, Brown RF, Smith AW. 1998. Isolation of reptilian calicivirus Crotalus type 1 from feral pinnipeds. J Wildl Dis 34:451–456.

Berke T, Golding B, Jiang X, Cubitt DW, Wolfaardt M, Smith AW, Matson DO. 1997. Phylogenetic analysis of the Caliciviruses. J Med Virol 52:419–424.

Berry ES, Skilling DE, Barlough JE, Vedros NA, Gage LJ, Smith AW. 1990. New marine calicivirus serotype infective for swine. Am J Vet Res 51:1184–1187.

Burkhardt W III, Blackstone GM, Skilling D, Smith AW. 2002. Applied technique for increasing calicivirus detection in shellfish extracts. J Appl Microbiol 93:235–240.

Culley AI, Lang AS, Suttle CA. 2003. High diversity of unknown picorna-like viruses in the sea. Nature 424:1054–1057.

Dean AG, Dean JA, Coulombier D, Brendel KA, Smith DC, Burton AH, Dicker RC, Sullivan K, Fagan RF, Arner TG. 1994. Epi Info, Version 6: A word processing, database, and statistics program for epidemiology on microcomputers. Atlanta, Georgia, Centers for Disease Control and Prevention.

Enserink M. 2000. Emerging diseases. Malaysian researchers trace Nipah virus outbreak to bats. Science 289:518–519.

Enserink M. 2003. SARS in China. China's missed chance. Science 301:294–296.

Evermann JF, Smith AW, Skilling DE, McKeirnan AJ. 1983. Ultrastructure of newly recognized caliciviruses of the dog and mink. Arch Virol 76:257–261.

Ganova-Raeva L, Smith AW, Fields H, Khudyakov Y. 2004. New Calicivirus isolated from walrus. Virus Res 102:207–213.

Green KY, Ando T, Balayan MS, Berke T, Clarke IN, Estes MK, Matson DO, Nakata S, Neill JD, Studdert MJ, Thiel HJ. 2000. Taxonomy of the caliciviruses. J Infect Dis 181:S322–S330.

Kurth A, Everman JF, Skilling DE, Matson DO, Smith AW. 2006. Prevalence of vesivirus in a laboratory-based set of serum samples obtained from dairy and beef cattle. Am J Vet Res 67:114–119.

Lang AS, Culley AI, Suttle CA. 2004. Genome sequence and characterization of a virus (HaRNAV) related to picorna-like viruses that infects the marine toxic bloom-forming alga Heterosigma akashiwo. Virology 320:206–217.

Matson DO. 1999. Re-analysis of serologic data from the Australian study of human health risks of infection by rabbit hemorrhagic disease virus. Royal Society of New Zealand, Wellington: Misc.-Series 55:62–66.

Matson DO, Berke T, Dinulos MB, Poet E, Zhong WM, Dai XM, Jiang X, Golding B, Smith AW. 1996. Partial characterization of the genome of nine animal caliciviruses. Arch Virol 141:2443–2456.

Meanger J, Carter MJ, Gaskell RM, Turner PC. 1992. Cloning and sequence determination of the feline calicivirus strain F9. Biochem Soc Trans 20:26S-.

Neill JD, Meyer RF, Seal BS. 1995. Genetic relatedness of the caliciviruses: San Miguel sea lion and vesicular exanthema of swine viruses constitute a single genotype within the Caliciviridae. J Virol 69:4484–4488.

Neill JD, Meyer RF, Seal BS. 1998. The capsid protein of vesicular exanthema of swine virus serotype A48: Relationship to the capsid protein of other animal caliciviruses. Virus Res 54: 39–50.

Poet SE, Skilling DE, Megyesl JL, Gilmartin WG, Smith AW. 1996. Detection of a non-cultivatable calicivirus from the white tern (Gygis alba rothschildi). J Wildl Dis 32:461–467.

Reid SM, Ansell DM, Ferris NP, Hutchings GH, Knowles NJ, Smith AW. 1999. Development of a reverse transcription polymerase chain reaction procedure for the detection of marine caliciviruses with potential application for nucleotide sequencing. J Virol Methods 82:99–107.

Rinehart-Kim JE, Zhong WM, Jiang X, Smith AW, Matson DO. 1999. Complete nucleotide sequence and genomic organization of a primate calicivirus, Pan-1. Arch Virol 144:199–208.

Seal BS, Lutze-Wallace C, Kreutz LC, Sapp T, Dulac GC, Neill JD. 1995. Isolation of caliciviruses from skunks that are antigenically and genotypically related to San Miguel sea lion virus. Virus Res 37:1–12.

Smith AW. 2000. Virus cycles in aquatic mammals, poikilotherms, and invertebrates. In: Hurst C, editor. Viral ecology. San Diego: Academic Press, pp 447–491.

Smith AW, Boyt PM. 1990. Caliciviruses of ocean origin: A review. J Zoo Wildl Med 21:3–23.

Smith AW, Prato CM, Skilling DE. 1977. Characterization of two new serotypes of San Miguel sea lion virus. Intervirology 8:30–36.

Smith AW, Vedros NA, Akers TG, Gilmartin WG. 1978. Hazards of disease transfer from marine mammals to land mammals: Review and recent findings. J Am Vet Med Assoc 173:1131–1133.

Smith AW, Skilling DE, Brown RJ. 1980a. Preliminary investigation of a possible lung worm (Parafilaroides decorus), fish (Girella nigricans), and marine mammal (Callorhinus ursinus) cycle for San Miguel sea lion virus type 5. Am J Vet Res 41:1846–1850.

Smith AW, Skilling DE, Dardiri AH, Latham AB. 1980b. Calicivirus pathogenic for swine: A new serotype isolated from opaleye Girella nigricans, an ocean fish. Science 209:940–941.

Smith AW, Skilling DE, Prato CM, Bray HL. 1981. Calcivirus (SMSV-5) infection in experimentally inoculated Opaleye fish (Girella nigricans). Arch Virol 67:165–168.

Smith AW, Mattson DE, Skilling DE, Schmitz JA. 1983. Isolation and partial characterization of a calicivirus from calves. Am J Vet Res 44:851–855.

Smith AW, Berry ES, Skilling DE, Barlough JE, Poet SE, Berke T, Mead J, Matson DO. 1998a. In vitro isolation and characterization of a calicivirus causing a vesicular disease of the hands and feet. Clin Infect Dis 26:434–439.

Smith AW, Skilling DE, Cherry N, Mead JH, Matson DO. 1998b. Calicivirus emergence from ocean reservoirs: Zoonotic and interspecies movements. Emerg Infect Dis 4:13–20.

Smith AW, Matson DO, Stein DA, Skilling DE, Kroeker AD, Berke T, Iversen PL. 2002a. Antisense treatment of caliciviridae: An emerging disease agent of animals and humans. Curr Opin Mol Ther 4:177–184.

Smith AW, Skilling DE, Matson DO, Kroeker AD, Stein DA, Berke T, Iversen PL. 2002b. Detection of vesicular exanthema of swine-like calicivirus in tissues from a naturally infected spontaneously aborted bovine fetus. J Am Vet Med Assoc 220:455–458.

Smith AW, Skilling DE, Castello JD, Rogers SO. 2004. Ice as a reservoir for pathogenic human viruses: Specifically, caliciviruses, influenza viruses, and enteroviruses. Med Hypotheses 63:560–566.

Studdert MJ. 1978. Caliciviruses. Brief review. Arch Virol 58:157–191.

Trampuz A, Prabhu RM, Smith TF, Baddour LM. 2004. Avian influenza: A new pandemic threat? Mayo Clin Proc 79:523–530.