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Characterization of San Miguel Sea Lion Virus populations using pyrosequencing-based methods

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ABSTRACT

San Miguel Sea Lion Virus (SMSV) is a small RNA virus in the genus *Vesivirus* with an unusually broad host range. Three populations of SMSV were examined by PCR amplification of the capsid precursor and putative helicase genes, followed by pyrosequencing. The populations were nasal swabs from two SMSV infected California sea lions (*Zalophus californianus*) from two different years, and a virus isolate from the earlier swab that was passaged in cell culture five times. In the capsid precursor, extensive deletions were prevalent in the passaged virus but uncommon in the clinical samples. A greater prevalence of point mutations was seen in the capsid precursor gene than in the putative helicase gene. In culture, the minority sequence in the capsid precursor at nucleotide position 5826 rapidly shifted after five passages to become the majority sequence. Levels of diversity at individual sites showed much more similarity between the two clinical samples than between the earlier clinical sample and the passaged culture from the same sample. SMSV appears to behave as a quasispecies. Assessment of original patient samples is preferable for understanding clinical SMSV populations.

1. Introduction

Due to lack of proofreading by their polymerases and constant selection by the host immune system, small RNA viruses have the fastest mutating genomes found in nature, and tracking evolution of these viruses is clinically relevant (Domingo and Gomez, 2007). High mutation rates together with asexual reproduction often results in these viruses behaving as quasispecies. In the traditional definition of a species, an organism evolves to become most fit for its ecologic niche. For small RNA viruses, it is more appropriate to examine clusters of related viruses, called quasispecies. If a single fittest sequence is not part of an overall group of related fit sequences, it can be at an evolutionary disadvantage (de la Torre and Holland, 1990). Small RNA viruses need to be able to change rapidly, and an individually fit virus that is unable to shift to other related fit viruses will be outcompeted. Therefore, a quasispecies does not evolve toward a single fittest genome sequence, and it is essential to understand population structure and mutation spectra of these viruses.

Caliciviruses are small positive stranded RNA viruses. There is evidence that feline calicivirus behaves as a quasispecies (Radford et al., 1998), and it is probable that all caliciviruses do so. San Miguel Sea Lion Virus (SMSV), a calicivirus in the genus *Vesivirus*, is one of the most commonly reported viruses of marine mammals (Van Bonn et al., 2000). It causes vesicular lesions in the mouth and on flippers of pinnipeds (Schaffer and Soergel, 1973; Smith et al., 1983b) and has been suggested as a cause of epizootic gastroenteritis of California sea lions (*Zalophus californianus*) (Schmitt et al., 2009). It is genetically indistinguishable from Vesicular Exanthema of Swine Virus (VESV), which is considered a reportable foreign animal disease (Neill et al., 1995). SMSV has a larger host range than other known vertebrate viruses, and has been found to infect numerous species including fish (Smith et al., 1980), amphibians, reptiles (Barlough et al., 1998), and primates (Smith et al., 1983a). There is evidence associating SMSV with vesicular lesions in humans (Smith et al., 1998a) as well as hepatitis (Smith et al., 2006). It is probable that SMSV is of marine origin, and fish hosts may play a significant role in propagation of disease (Smith et al., 1998b). Vesiviruses have demonstrated the ability to rapidly become more virulent. Feline calicivirus, which recently diverged from SMSV/VESV, remains stable in the marine environment (Kadoi and Kadoi, 2001) and capable of infecting California sea

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lions (Smith et al., 1998b). Strains of feline calicivirus have emerged that result in systemic hemorrhagic disease with 33–50% mortality (Pedersen et al., 2000). Feline calicivirus strains associated with hemorrhagic disease have arisen independently more than once (Coyne et al., 2006; Schorr-Evans et al., 2003).

Until fairly recently, all large-scale genome sequencing was done using the Sanger method, which produces several hundred bases of sequence per reaction. Recent technological advances in DNA sequencing dramatically improve the cost-efficiency of sequence production. The first of these technologies commercialized is pyrosequencing (Nordstrom et al., 2000), which produces up to a million sequences from a single reaction and requires less nucleic acid manipulation prior to sequencing, thus decreasing bias. When dealing with a small genome such as that found in caliciviruses, this enables a snapshot of an entire population, enabling the study of quasispecies. Pyrosequencing technology has been used to characterize variation in human immunodeficiency virus (Wang et al., 2007), hepatitis B (Lindström et al., 2004) influenza (Lackenby et al., 2008), and hepatitis C (Lahser et al., 2003) viruses. Use of next generation sequencing technologies may reduce sequencing costs of large data sets by more than two orders of magnitude, and costs are expected to decrease further as technologies advance (Li et al., 2009; Shendure and Ji, 2008).

The objectives of this study were to develop a protocol for assessing the population structure of SMSLV in clinical samples using pyrosequencing and to compare population structure from clinical samples with a cell-culture isolate of SMSV from one of the clinical samples.

2. Materials and methods

Nasal swabs from two confirmed calicivirus infected California sea lions (*Z. californianus*) (one from 2004 and one from 2006), and a virus isolate from the 2004 swab that was isolated onto BSC40 Vero cells and passaged five times were used for this study. Both animals were from the same managed collection with open water access. The duration of clinical signs and severity of SMSV infections during 2006 were greater than those during 2004.

Viral RNA was extracted from samples using the Qiagen Viral RNA Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Calicivirus-specific RT-PCR targeting the putative helicase and capsid precursor genes was then performed on the viral RNA using a modification of previously reported methods (Neill et al., 1995; Reid et al., 1999). Primers incorporating additional adaptor oligonucleotides "A" and "B" designed for 454 Life Sciences pyrosequencing were used (Pyro 1F 5′-GCCTCCCTCGCGCCATCAGGTGAGGTGTTTGAGAATTAG-3′, Pyro 1R 5′-GCCTTGCCAGCCCGCTCAGACATCAATTCCGCCAGACCA-3′, Pyro Hel1 5′-GCCTCCCTCGCGCCATCAGGTCCCAGTATTCGGATTTGTCTG-CC-3′, Pyro Hel2 5′-GCCTTGCCAGCCCGCTCAGAGCGGGTAGTTCAGT-CAAGTTCACC-3′). The PCR targeting the capsid precursor amplified approximately 728 bp plus primers from region A and part of region B, which form the N-terminal third of the capsid precursor (Reid et al., 1999). The PCR targeting the putative helicase amplifies approximately 307 bp plus primers. Together, these represent approximately 12.5% of the SMSV genome. RNA was reverse transcribed using the OneStep RT-PCR Kit (Qiagen, Valencia CA) at 50 °C for 30 min and then denatured at 94 °C for 5 min, followed by 36 cycles of denaturation at 94 °C for 30 s; annealing at 55 °C for 30 s, and extension at 72 °C for 60 s, with a final elongation step at 72 °C for 10 min. PCR products from both rounds were run in 1% agarose gels. Bands of interest were cut from the gel and extracted using the Qiaquick gel extraction kit (Qiagen). Products were quantified using a spectrophotometer (NanoDrop ND-1000, Thermo Fisher Scientific, Waltham, MA). To confirm product identity, Sanger sequencing was performed directly on aliquots using the Big-Dye Terminator Kit (Applied Biosystems, Foster City, CA) and analyzed on ABI 3130 automated DNA sequencers at the University of Florida Interdisciplinary Center for Biotechnology Research Sequencing Facilities. The 2004 oronasal swab sequences were used as reference sequences for pyrosequencing analysis.

For each sample, aliquots of 2.5×10^{11} copies each of the capsid precursor and putative helicase gene PCR products were combined. Samples were submitted for 454 sequencing. This was performed as described in the supplementary material and methods to Margulies et al. (2005) with slight modifications from the specifications (454 Life Sciences, Branford, CT). The purified PCR products were hybridized to DNA capture beads and clonally amplified by emulsion PCR. DNA capture beads containing amplified DNA were deposited in individual regions of a 4-region 25 mm × 75 mm PicoTiter plate, and DNA sequences determined with the GS-FLX instrument using the A primer. Individual DNA sequences were mapped to the SMSV reference sequences using Newbler (454 Life Sciences). The capsid precursor sequences were also manually double-checked using MUSCLE (Edgar, 2004), and multiple sets of 499 sequences from each helicase data set were manually checked for consistency with Newbler results. To reduce errors, we discarded homopolymer-associated indels with a prevalence of less than 3% from mutational analysis, and any mutation without a prevalence of 1% or greater in at least one sample. We also discarded sequences containing large deletions from mutational analysis. Indels resulting in a frameshift were not counted, as even if these sequences are non-erroneous they are not likely to produce functional virus.

Mutations with at least 1% prevalence of minority sequence in either the 2004 swab or the 2004 passaged culture sample that showed a 10-fold or greater difference in minority sequence prevalence with other sample were noted.

3. Results

Pyrosequencing of the original 2004 oronasal swab resulted in 13,939 helicase sequences and 599 capsid precursor sequences. Pyrosequencing of the five-passage culture of the 2004 oronasal swab resulted in 1418 helicase sequences and 220 capsid precursor sequences. Pyrosequencing of the original 2006 oronasal swab resulted in 5132 helicase sequences and 100 capsid precursor sequences. Sequence positions are given relative to a reference SMSV genome (GenBank Accession # U15301, Neill et al., 1995). Datasets were submitted to the SRA database at GenBank and are available under accession # SRP001023.

Results of deletion analysis are given in Table 1. Deletions in the capsid precursor region were more common than deletions in the putative helicase region. From the 2004 oronasal swab, 0.7% of the capsid precursor sequence amplification products contained significant deletions, whereas 67.7% of capsid precursor sequences from passage five of the 2004 culture had significant deletions, and 1% of the 2006 oronasal swab had significant deletions.

Results of mutational analysis are given in Table 2. In the putative helicase region, the first 231 nucleotides after the forward primer were used for mutational analysis, correlating with nucleotides 2424–2657 of the reference genome. There were eight mutations with at least 1% prevalence causing coding changes identified in the helicase region, and 17 prevalent noncoding mutations. The only helicase mutation with at least a 1.0% prevalence of minority sequence in the 2004 sample was at position 2424, a minority coding a substitution from glutamine to leucine. The minority population encoding a leucine substitution was more prevalent in the clinical sample (1.0%) than in the passaged culture (0.1%), and the 2006 clinical sample also displayed greater diversity than the culture (1.1%). The only helicase mutation with at least a 1.0% prevalence of minority

Table 1
Prevalence of large deletions in the three SMSV populations studied. Sequence positions are given relative to a reference SMSV genome (GenBank Accession # U15301).

Deletion	2004 swab	2004 culture	2006 swab
Helicase			
2424–2676	0%	0.07%	0%
2424–2680	0.02%	0.07%	0%
2434–2730	0%	0.07%	0%
Total	0.02%	0.21%	0%
Capsid			
5669–6386	0%	0.5%	0%
5670–6374	0%	2.3%	0%
5671–6336	0%	0.9%	0%
5671–6385	0%	13.2%	1%
5671–6390	0.2%	0.5%	0%
5672–6324	0%	0.5%	0%
5672–6327	0%	0.9%	0%
5672–6361	0%	3.2%	0%
5673–6394	0%	0.5%	0%
5683–6385	0%	1.8%	0%
5683–6391	0%	0.5%	0%
5684–6361	0%	1.4%	0%
5684–6364	0%	0.9%	0%
5686–6385	0%	7.7%	0%
5686–6390	0%	0.5%	0%
5691–6327	0%	1.4%	0%
5693–6385	0.3%	1.8%	0%
5693–6387	0%	0.5%	0%
5695–6328	0%	0.5%	0%
5696–6391	0%	0.5%	0%
5697–6385	0.2%	0%	0%
5697–6389	0%	0.5%	0%
5703–6383	0%	0.9%	0%
5699–6212	0%	0.5%	0%
5718–6385	0%	1.8%	0%
5719–6394	0%	1.8%	0%
5720–6324	0%	0.9%	0%
5720–6376	0%	0.5%	0%
5720–6379	0%	0.5%	0%
5720–6381	0%	1.8%	0%
5720–6383	0%	0.5%	0%
5722–6385	0%	0.5%	0%
5725–6390	0%	0.9%	0%
5734–6391	0%	0.9%	0%
5735–6384	0%	0.5%	0%
5736–6310	0%	1.4%	0%
5736–6355	0%	3.2%	0%
5747–6324	0%	0.5%	0%
5758–6388	0%	0.5%	0%
5758–6394	0%	0.9%	0%
5766–6296	0%	0.5%	0%
5778–6394	0%	0.5%	0%
5784–6394	0%	0.9%	0%
5787–6391	0%	0.5%	0%
5795–6382	0%	0.5%	0%
5797–6031	0%	0.5%	0%
5798–6394	0%	0.5%	0%
5809–6274	0%	0.5%	0%
5809–6341	0%	0.5%	0%
5825–6031	0%	0.5%	0%
5825–6394	0%	0.9%	0%
5831–6386	0%	0.5%	0%
5850–6341	0%	0.5%	0%
5881–6331	0%	1.4%	0%
5884–6329	0%	0.9%	0%
Total	0.7%	67.7%	1%

sequence in the 2004 passaged culture, at position 2632, was noncoding. Of the 24 majority differences between the 2004 and 2006 clinical samples, 17 were noncoding and seven were coding.

In the capsid precursor region, the first 226 nucleotides after the forward PCR primer were used for mutational analysis, correlating with nucleotides 5666–5891 of the reference genome. There were 34 mutations with at least 1.0% prevalence causing coding changes identified and 29 prevalent noncoding mutations. The most prevalent capsid precursor mutation identified in the 2004 sample, at position 5666, preceded the start codon. Eighteen of 22 (82%) prevalent capsid precursor mutations from the 2004 passaged culture caused coding changes. Of the 23 majority differences between the 2004 and 2006 samples, 20 were noncoding and three were coding. The three coding mutations were all relatively close, in the region from nucleotides 5793 to 5817. There were nine mutations with at least a 1.0% prevalence of minority sequence in either the 2004 swab or the 2004 passaged culture sample exhibiting a 10-fold or greater difference in minority sequence prevalence with the other sample. At nucleotide positions 5672, 5701, 5776, 5817, 5840, 5842, 5851, and 5879, there was 1.4–2.8% prevalence of minority sequence in the passaged culture sample and no minority sequence in the 2004 clinical sample. All of these sequence variants except for position 5817 resulted in coding changes. The 2006 clinical sample, like the 2004 clinical sample, had no sequence variability at any of these sites. At position 5826, a thymidine to guanine transversion resulting in a coding change from cysteine to tryptophan, there was 0.5% prevalence of thymidine in the 2004 clinical sample, 100% prevalence of thymidine in the 2004 passaged culture, and 0% prevalence in the 2006 clinical sample.

4. Discussion

Pyrosequencing is a useful approach for examining mutant spectra in virus populations (Eriksson et al., 2008). The diversity we report in San Miguel Sea Lion Virus (SMSV) and the population changes seen after short passage in culture show that SMSV behaves as a quasispecies. In culture, the minority sequence in the capsid precursor at position 5826 rapidly shifted after five passages to become the majority sequence. This mutation is in the carboxy-terminal A region of the capsid precursor, which, is cleaved off of the precursor by the viral protease when the mature capsid is formed (Sosnovtsev et al., 1998). In vesiviruses that have been examined, the A region is more conserved than the E region, but less than the B region (Glenn et al., 1999). Most antigenic epitopes are found in the E region, which is the most variable part of the genome and has been the most extensively examined in vesiviruses (Kreutz et al., 1998; Matsuura et al., 2001; Radford et al., 1998). We note that capsid precursor sites with amino acid coding differences between the 2004 and 2006 clinical samples (found from nucleotides 5793 to 5817) were close to 5826. Further work is needed to understand the biological significance of this region.

Overall, levels of diversity at individual sites showed much more similarity between the 2004 and 2006 clinical samples than between the 2004 clinical sample and the 2004 passaged culture. When diversity was found at a particular position in the 2004 clinical sample but not the passaged culture, we likewise found similar diversity in the 2006 clinical sample, despite the greater evolutionary distance between the two clinical samples. However, diversity present in the 2004 passaged culture but not the 2004 clinical sample was also absent from the 2006 clinical sample. This implies selection for diversity in California sea lions differs from that in cell culture.

An understanding of quasispecies provides information useful for clinical case management (Domingo and Gomez, 2007). Information on quasispecies structure is important to trace outbreaks. For many pathogens, serotyping has been found to correspond unreliably with relatedness (Matiz et al., 1998; Amonsin et al., 2002). Serotyping looks at immunogenic areas of the genome, which are under positive evolutionary selection. This is the sole criteria distinguishing VESV, a reportable foreign animal

Table 2
Mutational analysis of the three SMSV populations studied. Sequence positions are given relative to a reference SMSV genome (GenBank Accession # U15301). Mutations without a prevalence of 1% or greater in at least one sample are not shown. Sequences containing large deletions were excluded from mutational analysis.

Position	Nucleotide change	2004 swab	2004 culture	2006 swab	Coding change
Helicase					
2424	**A → T**	**1.0%**	**0.1%**	**1.1%**	**Gln → Leu**
2428	C → T	0.1%	0.3%	99.6%	No
2429	A → G	0.2%	0.1%	100.0%	Asn → Asp
2440	C → T	0.1%	0.1%	99.5%	No
2443	A → G	0.2%	0.4%	99.8%	No
2446	T → C	0.2%	0.2%	99.9%	No
2456	A → G	0.2%	0%	99.7%	No
2462	C → T	0.1%	0.1%	99.6%	No
2481	T → C	0.2%	0.1%	99.7%	No
2492	T → A	0.1%	0.1%	99.6%	Phe → Tyr
2496	T → C	0.1%	0.5%	99.8%	No
2497	T → C	0.1%	0.1%	99.8%	No
2499	G → C	0%	0%	99.6%	No if Δ @ 2497
2511	T → C	0.2%	0%	99.7%	Met → Thr
2512	G → A	0.1%	0.1%	99.7%	Met → Thr if Δ @ 2511
2521	T → C	0.2%	0.2%	99.6%	No
2523	C → T	0.3%	0.1%	99.6%	Ala → Val
2545	A → G	0.4%	0.3%	99.7%	No
2561	A → G	0.3%	0.2%	99.7%	Ile → Val
2575	T → C	0.2%	0.2%	99.7%	No
2577	G → A	0.1%	0.1%	100%	Ser → Asn
2593	G → A	0.1%	0.2%	99.6%	No
2614	A → G	0.2%	0.1%	99.7%	No
2632	T → C	0.5%	1.1%	99.7%	No
2635	C → A	0%	0%	100%	No
Capsid					
5666	C → T	3.5%	1.4%	3.0%	Noncoding
5672	T → C	0%	1.4%	0%	Met(Start) → Thr
5677	A → G	0.3%	1.4%	0%	Thr → Ala
5680	A → G	0.2%	0%	2.0%	Thr → Ala
5693	T → C	0%	0%	1.0%	Leu → Pro
5701	G → A	0%	1.4%	0%	Asp → Asn
5708	T → A	0%	0%	1.1%	Leu → His
5712	A → G	0%	0%	2.2%	No
5713	T → C	0.2%	0%	1.1%	Phe → Leu
5714	T → C	0.2%	0%	1.1%	Phe → Ser
5715	T → A	0%	0%	1.1%	Phe → Leu
5717	T → C	0.3%	1.4%	0%	Leu → Pro
5720	T → C	0.2%	1.4%	0%	Leu → Ser
5724	C → T	0.2%	0%	100%	No
5727	G → A	0.3%	0%	98.7%	No
5730	A → G	0.9%	2.8%	98.7%	No
5739	C → T	0.2%	0%	1.4%	No
5744	T → C	0.2%	1.4%	0%	Leu → Pro
5745	C → A	0%	0%	100%	No
5746	T → C	0.2%	0%	1.4%	Tyr → His
5749	G → A	0.3%	1.4%	1.4%	Gly → Arg
5763	C → T	0.7%	0%	100%	No
5766	A → C	0%	0%	100%	No
5769	C → T	0.2%	0%	1.6%	No
5776	G → A	0%	2.8%	0%	Val → Ile
5787	T → C	0.2%	1.4%	100%	No
5788	T → G	0%	0%	1.7%	Tyr → Asp
5793	A → T	0%	0%	100%	Glu → Asp
5796	C → T	0.2%	0%	98.3%	No
5800	C → T	0.2%	0%	100%	No
5802	T → A	0%	0%	100%	No
5810	T → A	0.5%	0%	100%	Val → Asp
5811	A → T	0%	0%	100%	No
5812	T → C	0.4%	0%	100%	No
5814	A → G	0%	0%	1.7%	No
5815–5817	GAT → gap	0%	0%	100%	Asp deleted
5817	T → C	0%	1.4%	0%	No
5819	A → G	0.5%	1.4%	0%	Asp → Gly
5826	**T → G**	**0.5%**	**100%**	**0%**	**Cys → Trp**
5826	T → C	0%	0%	100%	No
5831	A → G	0.7%	0%	1.7%	Asp → Gly
5838	T → C	0.5%	1.4%	0%	No
5840	T → C	0%	1.4%	0%	Phe → Ser
5842	A → G	0%	1.4%	0%	Asn → Asp
5844	C → T	0%	0%	100%	No
5849	G → A	0%	0%	5.1%	Cys → Tyr
5850	T → C	0.4%	0%	100%	No

Table 2 (*Continued*)

Position	Nucleotide change	2004 swab	2004 culture	2006 swab	Coding change
5851	T → A	0%	1.4%	0%	Phe → Ile
5853	C → T	0%	0%	96.6%	No
5853	C → G	0%	0%	3.4%	Phe → Leu
5854	T → C	0.5%	0%	1.7%	Ser → Pro
5859	C → T	0.2%	0%	100%	No
5864	A → G	0.4%	0%	8.5%	Tyr → Cys
5867	C → T	0%	0%	3.4%	Ala → Val
5868	T → C	0.2%	0%	1.7%	No
5871	T → C	0%	0%	1.7%	No
5872	A → G	0.2%	1.4%	0%	Ile → Val
5877	T → A	0.2%	0%	100%	No
5879	C → A	0%	1.5%	0%	Thr → Asn
5882	A → G	0.2%	1.5%	0%	Glu → Gly
5884	T → C	0.2%	1.5%	0%	Tyr → His
5886	C → T	0.2%	0%	100%	No
5889	T → C	0.2%	0%	100%	No

Specific positions of interest as discussed in the text are in bold.

disease in the US, from SMSV. Given the ability of caliciviruses to change rapidly, serotyping is likely to be fraught with problems for understanding marine caliciviruses or any quasispecies population structure, and it is probable that there are multiple undescribed serotypes. The antigenic regions of feline calicivirus in persistently infected cats have been shown to be hypervariable (Kreutz et al., 1998; Radford et al., 2003). There are at least 16 different serotypes of marine mammal caliciviruses. Although limited initial work has been done, the genetic relationship between serotypes is incompletely understood (Berke et al., 1997; Neill et al., 1995; Reid et al., 1999). Work examining diversity of vesiviruses outside of the hypervariable regions of the capsid has been limited to small numbers of sequences (Neill, 1992; Glenn et al., 1999; Foley et al., 2006).

The putative helicase region is much more conserved than the capsid precursor protein. This region is thought to be a helicase due to homology to the 2c protein of picornaviruses, although experimental evidence is lacking for this conclusion (Glenn et al., 1999). It is one of the most conserved regions in the vesiviruses (Glenn et al., 1999), implying a key biological role. Consistent with previous findings of conservation of this region, we report fewer differences between the 2004 clinical sample and the 2004 passaged culture than were found in the capsid. However, there were a greater number of potential coding differences seen in the putative helicase region than in the capsid precursor between the 2004 and 2006 clinical samples. One plausible explanation for this observation could be that the evolutionary relationships between our 2004 and 2006 samples differed in the helicase and capsid precursor protein regions. Recombination is not uncommon in those positive stranded RNA viruses that have been studied (Simmonds, 2006), and it is probable that this is also the case with SMSV. Further studies are needed to understand the significance of variation found in the putative helicase region.

Hosts play a significant role in virus evolution (Hitchman et al., 2007; van Hemert et al., 2007). SMSV/VES is a virus with an unusually large host range in a genus with a demonstrated ability to become highly virulent and lethal pathogens. Emerging disease is frequently associated with host switches. One recent meta-analysis of human diseases found that 816 of 1407 (58%) are zoonotic, and of human diseases, zoonotic diseases are significantly more likely to be emerging (Woolhouse and Gowtage-Sequeria, 2005). Most recent emerging diseases have been associated with host switches, including SARS coronavirus, H5N1 avian influenza, Hendra virus, Nipah virus, and AIDS. Further, viral diseases are much more likely to be emerging, especially RNA viruses (Woolhouse and Gowtage-Sequeria, 2005). Given the evidence of human infection with SMSV, further studies of the ecology and effects of host switching on the evolution of this virus are indicated (Smith et al., 1998a,b, 2006).

The large deletions in the 2004 cultured virus are consistent with what has been seen in a number of viruses in culture at a high multiplicity of infection (Huang and Baltimore, 1970). Defective virus, known as Von Magnus particles, survive and passage successfully when normal virus is also present to provide complementing functional proteins. While smaller RNA has been found in feline calicivirus, it was found to be normal subgenomic RNA packaged in the virion (Neill, 2002).

Potential sources of error in our protocol may result from reverse transcription, polymerase chain amplification, and emulsion PCR and pyrosequencing. The reagents used in this study for reverse transcription and PCR amplification (Qiagen OneStep RT-PCR Kit) have been used previously in conjunction with pyrosequencing to look for mutations in influenza neuraminidase genes (Lackenby et al., 2008). With the Qiagen reagents used, reverse transcriptase error rates are expected to be approximately 1 in 10,000, and DNA polymerase error rates are 2–3 per 100,000 (Qiagen). Error rates with an earlier model of pyrosequencer (GS20) have been found to be approximately 0.5% (Huse et al., 2007). The majority of pyrosequencing errors are associated with errors in reading lengths of homopolymers (Huse et al., 2007). Use of high fidelity DNA polymerases is expected to reduce PCR errors and reduce error rates; however, the higher error rate of reverse transcription, which precedes PCR amplification, is a greater concern. One method to improve true mutation detection in future studies would be to run duplicate or triplicate samples. Replicates could be distinguished from each other by inserting short sequence "barcodes" in the PCR primers between the pyrosequencing linkers and the calicivirus-specific sequence (Hoffmann et al., 2007). Mutations only prevalent in one replicate would be likely due to reverse transcription/PCR errors.

There was a marked disparity in the number of resultant sequences from the helicase and capsid precursor. Two possible explanations include quantitation errors prior to pyrosequencing and pyrosequencing bias against the capsid precursor PCR product, which was longer than the desired length for a standard emulsion PCR template. The authors feel that the second explanation is more probable. Significant bias against DNA fragments over 400 bp in pyrosequencing has been previously demonstrated and attributed to suboptimal emulsion PCR of longer fragments (Torres et al., 2008). If significant bias against longer capsid precursor products was present, then bias toward large deletions present may result in overrepresentation, and the proportion of large deletions in the actual samples may not be as great. However, this would not affect the presence of differences between the samples.

The differences between the 2004 clinical sample and the same virus after only five passages in cell culture have significant implications for studies done using isolated virus. It has previously been noted that SMSV isolates often take three to six blind passages before exhibiting cytopathic effects (Smith and Boyt, 1990). We have shown that population changes can occur in this evolutionary distance, and assessment of original patient samples is preferable for understanding SMSV populations. Given the unusually broad host range of SMSV, this virus may serve as a good model in future studies for understanding quasispecies evolution across host switches.

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