AN ABSTRACT OF THE THESIS OF

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Alvin W. Smith

The Caliciviridae contains many viruses which are pathogenic to humans, marine mammals, domestic animals, and numerous species of wildlife. Currently there is no single assay to detect antigenic response to the multiple serotypes of calicivirus. The development of calicivirus specific synthetic peptides having highly conserved epitopes common to many serotypes would facilitate the development of a simple and rapid serologic assay for calicivirus antibodies irrespective of the serotype. Calicivirus cDNA recombinants which express fusion proteins that react with multiple calicivirus typing sera may be useful in the development of a serologic assay for antibodies to caliciviruses. For this purpose RNA was isolated from cell culture infected with San Miguel sea lion virus type 5 (SMSV-5) and used to construct a cDNA library, named SMSV-5 lambda. Immunoassay techniques were used to screen the SMSV-5 lambda library and a second cDNA library, named SMSV-5RT, also constructed from SMSV-5. One recombinant named 8-SN was identified which produced a fusion protein that reacted positively with a pool of four polyclonal calicivirus typing sera (SMSV-5, SMSV-13, SMSV-15, and SMSV-17). This construct was amplified, induced, and a fusion protein identified which reacted positively in four western blot assays using individual polyclonal typing sera to the caliciviruses SMSV-13, SMSV-15, SMSV-16, and SMSV-17.

Calicivirus Recombinant Expressing Fusion Protein Reacting to Multiple Calicivirus Typing Sera

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Dean of Graduate School

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Michael A. Stone

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INTRODUCTION

Historical

In 1932 in Orange County California, swine ranchers observed vesicular lesions on the feet and dorsal nasal regions of their pigs that were being fed raw garbage. The disease was initially diagnosed as foot and mouth disease (FMD); consequently, all infected materials were destroyed.[10,31,38,43] Following additional outbreaks and further investigation, FMD virus was ruled out and a new viral disease was identified. The virus responsible was named vesicular exanthema of swine virus (VESV), later to be classified as a member of the Caliciviridae family.[29,31,38] VESV is clinically indistinguishable from FMD virus.[10,38,43] By 1953, VESV had spread to 46 states resulting in the initiation of an emergency eradication program by the Bureau of Animal Industry. The last reported outbreak occurred in Secaucus, New Jersey in 1956, and in 1959 the USDA declared that VESV was eradicated from the United States, and classified it as a foreign animal disease.[3,38]

In 1972, Smith and coworkers were investigating premature parturition in California sea lions (*Zalophus californianus*) on San Miguel Island off the southern California coast, and subsequently isolated a calicivirus from an aborting animal.[10,39] The virus isolated was named San Miguel sea lion virus (SMSV).[10,39] This was the first isolation of a calicivirus from a pinniped.[38] Interestingly, when this serotype was inoculated into swine it produced a clinical disease identical to that of VESV.[7,39] This

new viral isolate, which is presently classified as a calicivirus, was indistinguishable from VESV using animal infectivity, physiochemical and morphological means.[38,39]

Host Range and Pathogenicity

Caliciviruses and calicivirus-like viruses vary in their pathogenicity and infect a broad range of species.[3,10,29] For example, caliciviruses have been either detected and/or isolated from cats, dogs, swine, bovine, fish, reptiles, rabbits, marine mammals, and humans.[1,2,6,12,33,35,36,38,41] Caliciviruses have been demonstrated to be blistering agents capable of inducing vesicular lesions on the hoofs and snouts of swine, nasoral lesions and upper respiratory disease in cats, vesicular lesions and erosion of the non-haired areas in pinnipeds, fatal hemorrhagic disease in rabbits, acute non-bacterial gastroenteritis in humans, and a sometimes fatal hepatitis E in humans.[2,4,6,10,11,13,15,17,36,41]

Characteristics of Calicivirus

Caliciviruses are non-enveloped, non-segmented, single stranded, positive sense, polyadenylated RNA viruses. The genome is 7.5-8Kb in length and has a VpG protein attached by a phosphodiester linkage on the 5' end, and the VpG protein is required for infectivity.[5,10,16,21,22] An important characteristic of caliciviruses in regard to this study is the presence of a 3' co-terminal nested set of sub-genomic viral RNAs, which are found in the cytoplasm of infected cells.[8,9,22] Near the 3' end there is a 2.4Kb open reading frame.[20] Recent studies have demonstrated an abundance of a 2.4Kb sub-genomic RNA in calicivirus infected cells which has been shown to code for a

single polypeptide.[21] The capsid is comprised of multiple repeating units of a single polypeptide generated from the 2.4Kb sub-genomic RNA.[8,20] Morphologically caliciviruses are 36nm in diameter, have a sedimentation coefficient of 183S in a sucrose gradient, and by electron microscopy show 22 cup-like depressions, or calyces, on their capsid surface.[28,30,45]

Project Objective

The Caliciviridae family contains more than 42 known serotypes divided into five groups.[18] Many of these agents are pathogenic to humans, pinnipeds, companion animals, livestock and numerous other species.[3] Multiple identical repeating units of a single polypeptide are thought to make up the capsid protein of all caliciviruses. This, along with some highly conserved genomic regions, suggests that there are multiple conserved epitopes common to many or all caliciviruses.[5,8,20,31] The synthetic development of common conserved antigenic peptides would facilitate the development of a simple, rapid, serological assay for caliciviruses irrespective of the serotype.

The objective of this project was to isolate a clone containing calicivirus cDNA which when induced would produce a detectable polypeptide common to many serotypes of the Caliciviridae family, but unique to the Caliciviridae family. This was accomplished in three phases. First, a representative cDNA library of the calicivirus serotype SMSV-5 was constructed and ligated into a lambda vector system capable of both prokaryotic and eukaryotic expression.[37,42] The serotype SMSV-5 was chosen due to its broad host range, ability to replicate in cell culture, and the existence of a plasmid cDNA library, SMSV-5RT.[23] Second, the cDNA library, SMSV-5RT, along with the new SMSV-5 lambda library were screened with a panel of polyclonal antibodies against SMSV-5,

SMSV-13, and SMSV-17. Plaques and/or clones which demonstrated the highest antigenicity were selected and plaque/colony purified. Third, the selected plaques or colonies were amplified and expressed, and the fusion protein was isolated and analyzed via western blot against a different pool of polyclonal antibodies, SMSV-14, SMSV-15, and SMSV-16.[40]

MATERIALS AND METHODS

The major steps from infecting cell culture with SMSV-5 to identification of the fusion protein are outlined in Figure 1.

FIGURE 1

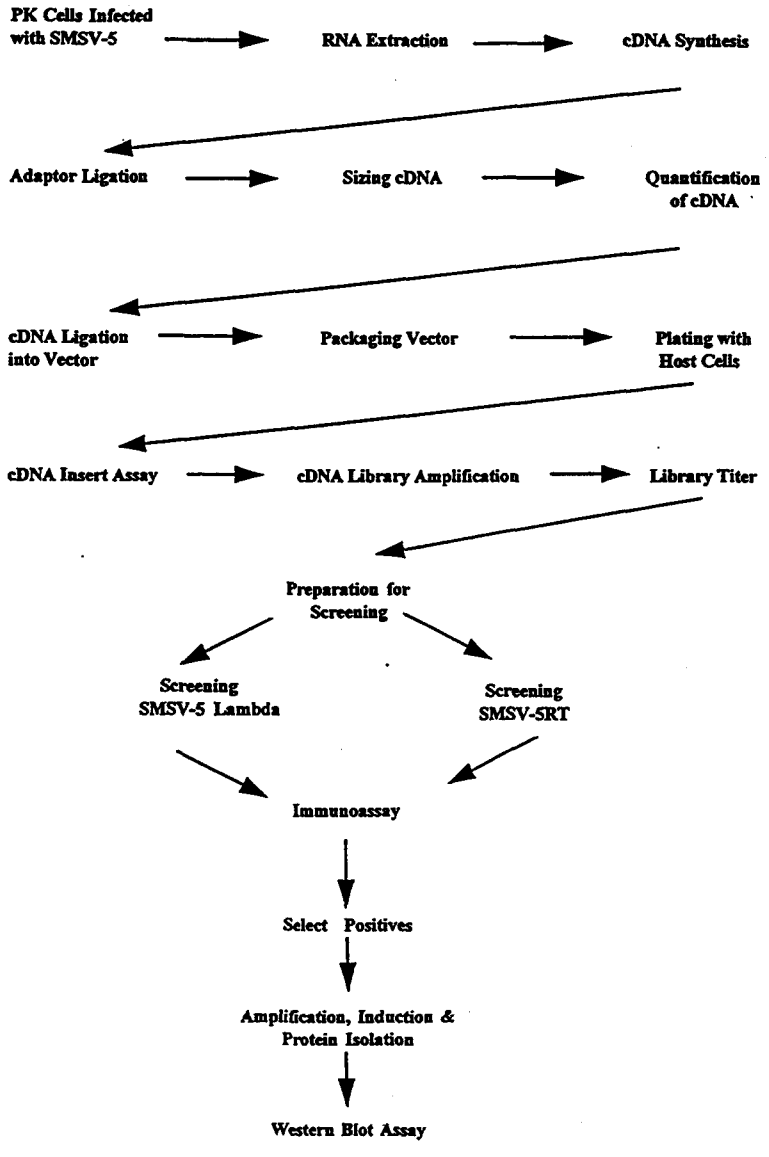

Figure 1 Flow chart starting with SMSV-5 infected PK cells to the purification and detection of a positive fusion protein.

SMSV-5 RNA Extraction

SMSV-5 RNA was isolated and purified as follows. Porcine kidney (PK) cells (American Type Culture Collection PK 15) were grown in a 75 cm^2 tissue culture flask with 30ml of Eagle's minimum essential medium supplemented with 5% bovine calf serum, 0.1mg/ml streptomycin, 0.5mg/ml gentamicin sulfate, 100U/ml penicillin and 2mM L-Glutamine at a temperature of 37°C until a monolayer of confluent cells formed. The medium was removed and a 1:10 dilution of a $4x10^7$ $TCID_{50}$/ml frozen stock of SMSV-5 (supplied by the Laboratory for Calicivirus Research, Oregon State University) was added to make a total volume of 10ml. The tissue culture flask was incubated for one hour at 37°C to allow the virus time to attach to the cells. The medium was removed and replaced with 10ml of fresh tissue culture medium and incubated overnight at 37°C. Within 24 hours all of the cells were lysed and/or free floating in the medium as determined by microscopic examination. The flask was frozen overnight at -70°C to disrupt the cell membrane and release the virus. The following morning, the contents of the flask were removed in 750ul aliquots and added to an equal volumes of 1,1,2-trichloro-1,2,2-trifluoroethane (Uvasol) in sterile 1.5ml microcentrifuge tubes. The samples were vigorously mixed for 10 seconds followed by 10 seconds on ice for a total of five cycles, and then centrifuged at room temperature at 10,000xg for 5 minutes. The top 700ul aqueous layer was removed to a fresh tube and stored at -70°C.

Two aliquots of the Uvasol extracted samples were thawed on ice and added to an equal volume of 16% polyethylene glycol 6000-8000 (2X PEG) in solution, and 0.8M NaCl in diethylpyranocarbonate (DEPC) treated water. The reaction was mixed, incubated for 30 minutes at 4°C, and then centrifuged at 10,000xg for 15 minutes to pellet the protein aggregates. The supernatant was removed and the pellet gently re-suspended in 150ul of 0.1M Tris pH 7.5, 12.5mM disodium ethylenediaminetetra-

acetate (EDTA), 0.15M NaCl, and 1% w/v sodium dodecyl sulfate (SDS) (1x proteinase K buffer), and then mixed at low speed for 15 minutes. The contents of the two tubes were pooled to make a total volume of 300ul.

Protein was digested by adding 120ug of freshly thawed proteinase K to the sample (Gibco BRL) The contents were gently mixed and incubated for 30 minutes at 37°C. Following the incubation, 50ul of 10% cetyltrimethlammonium bromide (CTAB) dissolved in DEPC treated water and 50ul of 4M NaCl were added, and the sample was mixed and then incubated at 56°C for 30 minutes. Protein was extracted from the sample with 420ul of 1:1 phenol:chloroform (Gibco BRL) by vigorously mixing for 2 minutes and then centrifuging at 10,000xg for 10 minutes at room temperature. A 320ul aliquot of the top aqueous layer was removed and added to an equal volume of chloroform in a fresh tube, again vigorously mixed for 2 minutes and centrifuged at 10,000xg for 10 minutes. A total of 240ul of the top aqueous layer was removed to a fresh tube.

Precipitation of the RNA was accomplished by adding 16ul of 3M sodium acetate and 640ul of ice cold 100% ethanol. The sample was vigorously mixed for 10 seconds, and then incubated overnight at -20°C. In the morning, the RNA was pelleted by centrifugation at 10,000xg 4°C, for 15 minutes. The supernatant was discarded, the pellet was washed with ice 70% cold ethanol and centrifuged for 10 minutes at 10,000xg at room temperature. The 70% ethanol was removed and then the pellet was air dried. The pellet was then re-suspended in 55ul of DEPC treated water. From the sample, 5ul was removed and added to 95ul of DEPC treated water and tested for RNA concentration and purity by spectrophotometry using a Beckman DU spectrophotometer equipped with nucleic acid software (OD 260/280 ratio). The remaining 50ul of sample was stored at -70°C.

cDNA Synthesis

The cDNA library was constructed using the Zap Express cDNA Synthesis Kit which is based on the Gubler Hoffman method, and the Zap Express cDNA Gigapack II Gold Cloning Kit (Stratagene, La Jolla, California). The protocol provided by Stratagene was followed with minor changes to meet our specific needs (Figure 1)[42]

Five ul of purified RNA (1.25ug/ul), containing both viral RNA and cellular mRNA (from the PK cells), was added to 12.5ul of 0.1M methyl mercuric hydroxide (MeHgOH). The sample was gently mixed, and incubated at 56°C for 7 minutes to relax the secondary structure of the RNA.[26] First strand synthesis was accomplished by adding in order to a fresh, RNase free tube the following: 5.0ul of 10X first strand buffer, 3.0ul of first strand methyl nucleotide mixture (10mM dATP, dGTP, and dTTP and 5mM 5-methyl dCTP) 2.0ul linker-primer, (oligo dT linker-primer with an *Xho* I site) 18ul of DEPC treated water and 2.0ul of RNase block ribonuclease inhibitor. The contents of the tube were mixed, added to the 17.5ul of the MeHgOH treated RNA, mixed again, and incubated at 25°C to allow the primer and template to anneal. Following the 10 minute incubation, 2.5ul of Stratascript RNase H-reverse transcriptase (100U/ul) was added to the reaction tube. The contents of the tube were gently mixed, briefly centrifuged to pool the material, and then incubated for 1 hour at 37°C. After one hour of incubation at 37°C the reaction was placed on ice.

Second strand synthesis was accomplished by adding in order to the first strand reaction tube the following components: 20ul of 10X second strand buffer, 6ul of second strand nucleotide mix (10mM dATP, dGTP and dTTP and 26mM dCTP) and 103.2ul of sterile double distilled (dd) water. Rapidly, 3.0ul of RNase H and 17.8ul of DNA polymerase I were added to the reaction tube. The components were gently mixed, briefly centrifuged, and incubated at 16°C for 2.5 hours and then stored on ice.

The cDNA terminal ends were blunted and/or filled in by adding 23ul of dNTP mix (2.5mM dATP, dGTP, dTTP, and dCTP) and 2.0ul of *Pfu* DNA polymerase. The contents of the reaction tube were quickly mixed, briefly centrifuged, and incubated at 72°C for 30 minutes. The synthesized cDNA was protein extracted as before with an equal volume of phenol:chloroform, followed by an equal volume chloroform extraction. Fifteen ul of the upper aqueous layer was removed to a fresh tube containing 20ul of 3M sodium acetate and 400ul of ice cold 100% ethanol. The components were gently mixed and the cDNA was precipitated by overnight incubation at -20°C. In the morning, the cDNA was pelleted by centrifugation at 10,000xg and 4°C, for 60 minutes. The supernatant was removed, the pellet was gently washed with 500ul of ice cold 70% ethanol, and then centrifuged for 2 minutes at 10,000xg at room temperature. The ethanol was aspirated and the pellet air dried.

Adaptor Ligation

The cDNA pellet was re-suspended in 9ul of *Eco*R I adaptors (0.4ug/ul). Ligation of the adaptors to the cDNA was accomplished by adding in order the following components: 1ul of a solution consisting of 50mM Tris pH 7.5, and 70mM $MgCl_2$ (10x buffer #3), 1ul of rATP, and 1ul of T4 DNA ligase (4 U/ul). The components were very gently mixed, briefly centrifuged and incubated at 8°C for 48 hours. The DNA ligase was then heat inactivated by incubating at 70°C for 30 minutes. The reaction tube was briefly spun to pool the contents and then allowed to cool to room temperature. Kinasing of the *Eco*R I ends of the cDNA was accomplished with the addition of 1ul of 10x buffer #3, 2ul of 10mM rATP, 6ul of double distilled (dd) water, and 10ul of T4 polynucleotide kinase (10U/ul). The contents were gently mixed and incubated for 30 minutes at 37°C, and then the kinase was heat inactivated at 70°C for 30 minutes. The

reaction tube was briefly spun and allowed to cool to room temperature. *Xho* I digestion was accomplished with the addition of 28.0ul of 10x buffer #3 and 3.0ul of *Xho* I (40U/ul) to the sample which was then gently mixed and incubated at 37°C for 1.5 hours. The reaction was cooled to room temperature and 5ul of a solution consisting of 1M NaCl, 200mM Tris pH 7.5 and 100mM EDTA (10X STE buffer) was added. The last three steps result in cDNA fragments with a *Eco*R I adaptors on the 3' end and *Xho* I adaptors on the 5' end.

Sizing of cDNA Fragments

Separation of the larger cDNA fragments from the excess adaptors and small cDNA fragments was accomplished by centrifugation at 400xg for 2 minutes through a Sephacryl S-400 spin column. Four fractions were collected. Each fraction was phenol-chloroform extracted to remove the kinase. The upper aqueous layer was removed to a fresh tube. Two volumes of ice cold 100% ethanol were added to each fraction, mixed, and incubated at -20°C overnight to precipitate the cDNA.

The following morning the samples were centrifuged at 10,000xg for 60 minutes at 4°C. The supernatant was aspirated and the pellet was washed with 200ul of ice cold 80% ethanol. The samples were centrifuged for 2 minutes at 10,000xg to pellet the cDNA, the ethanol was removed, and the pellets were then air dried. Each pellet was re-suspended in 3ul of dd water.

cDNA Quantification

The quantity of the cDNA was estimated with an ethidium bromide assay plate (0.8% w/v agarose, Tris-acetate medium and 1ug 3,8-diamino-6-ethyl-5-phenylphenanthridium bromide). Linear DNA standards (Gibco BRL) were prepared of: 500ng/ul, 250ng/ul, 125ng/ul, 60ng/ul, 30ng/ul 15ng/ul and 7ng/ul concentrations. Each standard (0.5ul) was spotted onto a labeled location on an assay plate immediately followed by spotting 0.5ul of each of the four cDNA fractions onto a labeled plate location. The samples were allowed to absorb ethidium bromide for 15 minutes and then the plate was inverted and photographed under UV light. Estimates of the quantity of cDNA was made by comparing the amount of fluorescence of the samples to the known DNA standard values. The cDNA fractions were pooled and estimated to have a concentration of 50ng/ul with a total volume of 10ul.

Vector Ligation

From the 10ul sample of cDNA, 2ul was removed for test ligation into the vector and the remainder was stored at -20°C. The following three ligation reaction mixes were prepared: 1) A vector only control ligation containing 1ug of vector arms, 2.5ul of double distilled (dd) water, 0.5ul of 10X buffer #3, 0.5ul of 10mM rATP, and 0.5ul of T4 DNA ligase. 2) A test DNA ligation control containing 1ug of vector arms, 16ul of the Zap kit test insert DNA, 05ul of 10X buffer #3, 0.5ul of 10mM rATP, 0.9ul of dd water, and 0.5ul of T4 DNA ligase. 3) A 100ug sample cDNA ligation containing 1ug of vector arms, 2ul of the sample cDNA, 0.5ul of 10X buffer #3, 0.5ul of 10mM rATP, 0.5ul of dd water, and 0.5ul of T4 DNA ligase. All three reactions were incubated for 48 hours at 4°C.

Vector Host Cells

Twenty four hours prior to packaging the ligated cDNA, two host strains of *E. coli*, XL1-Blue MRF' and VCS257 (Stratagene) were inoculated into LB media supplemented with 10mM $MgSO_4$ to and 0.2% maltose. The cultures were grown at 37°C to mid log phase, and the cells centrifuged at 400xg for 10 minutes. The supernatant was carefully decanted and the cells re-suspended in 10mM $MgSO_4$ to an OD_{600} of 0.5 using a Gilford Spectrophotometer 260.

Packaging Reaction

Four packaging reactions were completed with (1) ligated sample cDNA, (2) ligated Zap Kit test DNA, (3) vector only DNA, and (4) a positive control wild lambda I857 Sam 7 DNA. To each partially thawed packaging extract (Stratagene), 1ul each of the 4 samples was added respectively. Rapidly, 15ul of sonic extract (Stratagene) was added to each reaction tube, the contents were very gently mixed, and then incubated for 105 minutes at 22°C. The reactions were stopped by adding 500ul of a solution comprised of 0.1M NaCl, 8.0mM $MgSO_4$ $7H_2O$, 0.5M Tris, and 0.01% w/v gelatin pH 7.5 (SM buffer) and 20ul of chloroform. The reactions were then gently mixed and briefly spun to sediment the debris.

Plating with Host Cells

The packaged ligated products were plated in the following five trials. 1) A 1ul aliquot from the experimental cDNA sample, and 2) 1ul of a 1:10 dilution from the

experimental cDNA sample were added to separate microcentrifuge tubes containing 200ul of fresh XL1-Blue MRF' cells. 3) A 1ul aliquot of the vector only, and 4) 1ul of the Zap Kit test DNA were added to separate microcentrifuge tubes containing 200ul of fresh XL1-Blue MRF' cells. 5) A 10ul aliquot of the positive control diluted 1:10,000 in SM buffer was mixed with 200ul of fresh VCS 257 cells in a microcentrifuge tube. All five cultures were incubated at 37°C for 15 minutes. Five ml of 48°C 0.7% NZY top agarose (0.09M NaCl, 8.0mM $MgSO_4$ 7·H_2O, 0.5% w/v bacto-yeast extract, 1% w/v cassein hydrolysate, pH to 7.0 with 5N NaOH, 0.7% w/v agarose) was aliquoted into five 15 ml polypropylene tubes. To four of the tubes, 15ul of 0.5M isopropyl-B-thio-galatopyranosidase (IPTG) was added and mixed, followed by the addition of 25ul of 250mg/ml 5-bromo-4-chloro-3-indoyl-B-D-galactopyranoside (X-gal) and mixed, and then maintained at 48°C. The contents of the experimental sample tubes, the vector only tube, and the test Zap Kit DNA ligation tube were added to separate tubes containing the 5ml of 48°C NZY top agarose, IPTG, and X-gal. The tubes were quickly mixed and plated onto pre-warmed NZY agar plates. The contents of the positive control tube were mixed with 5ml of 48°C NZY top agarose and immediately plated on to a pre-warmed NZY plate. The plates were cooled, inverted, and incubated overnight at 37°C.

cDNA Insert Assay

The pBK-CMV phagemid was isolated in the following manner. Several of the positive white plaques on the sample plate were cored with a pasture pipette and placed into individual 1.5ml microcentrifuge tubes containing 500ul of SM buffer and 20ul of chloroform. The tubes were vigorously mixed to disrupt the core plug, and then incubated at 4°C overnight to allow the diffusion of the phage particles into the buffer. In the interim, fresh XL1-Blue MRF' was inoculated into 50ml of LB media supplemented with $MgSO_4$ and 0.2% maltose, and XLOLR (Stratagene) was inoculated into

LB media; both were incubated overnight at 37°C. In the morning, 0.5ml of each culture was inoculated into 50ml of fresh LB media. The media for the XL1-Blue MRF' strain was supplemented with $MgSO_4$ and 0.2% maltose. The XL1-Blue MRF' strain was grown to mid log phase, centrifuged for 15 minutes at 400xg, the supernatant was decanted, and the cells were re-suspended in 10mM $MgSO_4$ to an OD_{600} of 0.5. A mix of 250ul of the phage stock, 200ul of XL1-Blue MRF' cells, and 1ul of ExAssist helper phage (Stratagene) was made in a 1.5ml centrifuge tube for each phage supernatant sample, and incubated at 37°C for 15 minutes to allow the phage to attach to the cells. The contents of each tube was transferred to a fresh labeled 6ml polystyrene tube containing 3ml of 37°C LB media. The tubes were incubated overnight at 37°C with shaking. In the morning, the tubes were incubated at 70°C to inactivate the cells, followed by centrifugation at 400xg for 15 minutes to pellet the cellular debris. The supernatant, containing pBK-CMV phagemid packaged as filamentous phage particles, was transferred to a fresh tube and stored at 4°C.

For each of the four phagemid samples, two 1.5ml microcentrifuge tubes were loaded with 200ul of XLOLR cells grown to an OD_{600} of 1.0. Ten ul and 100ul of the pBK-CMV phagemid supernatant was added to each of the two tubes, respectively. The samples were incubated for 15 minutes at 37°C, 300ul of LB broth was added, and the tubes were then incubated for an additional 45 minutes. Two hundred ul from each microcentrifuge tube was individually transferred to 3ml of 48°C LB top agarose and plated onto pre-warmed LB plates containing 50ug/ml kanamycin. The plates were cooled, inverted, and incubated at 37°C overnight. Isolated colonies were picked and inoculated into LB media containing 50ug/ml kanamycin and incubated overnight at 37°C. In the morning 1.5ml from each sample was decanted into a fresh microcentrifuge tube, centrifuged at 10,000xg for 30 seconds and most of the supernatant was discarded leaving approximately 75ul in the tube. The remaining liquid and pellet was re-suspended by vigorous mixing. To disrupt the cell membrane 300ul of a solution of

10mM Tris pH 7.5, 1mM EDTA, 0.1N NaOH, and 0.5%SDS (TENS) was added to each tube followed by 2 to 5 seconds of mixing. Cellular debris and chromosomal material were separated by adding 150ul of 3M sodium acetate. The contents were thoroughly mixed and then centrifuged for 2 minutes at 10,000xg. The supernatant containing the phagemid DNA was decanted to a fresh tube containing 900ul of ice cold 100% ethanol. The contents were well mixed and then incubated at -70°C for one hour to allow the precipitation of the nucleic acids. The nucleic acids were pelleted by centrifugation at 10,000xg for 15 minutes at room temperature. The supernatant was discarded and the pellet was washed 2 times with 1000ul of ice 70% cold ethanol. The ethanol was removed and the pellet was re-suspended in a solution of 10mM Tris pH 8.0 and 1mM EDTA, pH 8.0 (TE buffer pH 8.0).

A 7% agarose gel containing ethidium bromide at a concentration of 0.5ug/ml was run to determine the presence of cDNA inserts in the extracted pBK-CMV phagemid.[25] This was accomplished using an 8 lane mini gel apparatus and power supply (Bio-Rad). Five ul from selected pBK-CMV phagemid extracts were mixed with 4ul of a solution 100mM Tris pH 6.8, 200mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol (2X SDS loading buffer), and 10ul of a solution of 0.04M Tris-acetate and 0.001M EDTA (TAE buffer), and then loaded into 7 of the 8 wells of the gel.[24] The remaining well was loaded with 1ul of super coiled DNA ladder (BRL), 4ul of 2X loading buffer and 10ul of TAE buffer. The gel was run at 80 volts using 1X TAE as the running buffer. The bands were visualized with UV light and photographed.

cDNA library Amplification

The remaining 4ul of ligated cDNA was packaged in two 1ul reactions and one 2ul reaction as previously outlined. The resulting primary libraries were immediately amplified as follows. Two hundred and twenty five ul of each packaging reaction was

combined in a 1.5ml microcentrifuge tube with 600ul of fresh log phase XL1-Blue MRF' cells supplemented with $MgSO_4$ and 0.2% maltose. The tubes were incubated for 15 minutes at 37°C to allow the phage to attach to the cells, and then the contents of each tube were individually added to 6.5ml of 48°C NZY top agarose. The tubes were quickly mixed and immediately plated on to pre-warmed NZY plates. The plates were cooled, inverted, and incubated until the plaques reached 1 to 2 mm in diameter. They were then overlaid with 8ml of SM buffer and incubated at 4°C overnight to allow the phage to diffuse into the buffer. In the morning the cold plates were gently shaken for 5 minutes at room temperature. The buffer from each plate was collected and pooled into a 50ml Falcon polypropylene tube. Chloroform was added to a concentration of 5.0%. The contents were well mixed and then incubated for 15 minutes at room temperature. Cellular debris was removed by centrifugation at 500xg for 10 minutes. The supernatant was decanted to a fresh tube and chloroform was added to a final concentration of 0.3%. Dimethylsulfoxide (DMSO) was added to a final concentration of 7.0%. The amplified library was aliquoted into 1.5 ml cryo-tubes (Corning) and stored at -70° C.

cDNA Library Titering

A sample of the amplified library was titered as follows. Serial dilutions of a 100ul sample of the library were made from $1x10^{-1}$ to $1x10^{-9}$. Using 1.5ml microcentrifuge tubes, 100ul of each dilution was incubated with 200ul fresh log phase XL1-Blue MRF' cells for 15 minutes at 37°C. The contents of each reaction tube was then added to 3ml of 48°C top agarose, mixed, and plated onto pre-warmed NZY agar plates. The plates were cooled, inverted, and incubated overnight at 37°C.

Screening

The SMSV-5RT and the lambda SMSV-5 libraries were processed for screening separately as indicated below, and were then simultaneously screened with an immunological assay also as outlined below.

Lambda SMSV-5 Library Processing For Screening

The lambda SMSV-5 library was prepared for immunological screening in the following manner. One hundred ul aliquots of the cDNA-SMSV-5 library containing approximately $2x10^4$ pfu were incubated with 200ul of log phase XL1-Blue MRF' cells for 15 minutes at 37°C to allow the phage to attach to the cells. The contents of each reaction were added to 3ml of 48°C NZY top agarose, quickly mixed, and poured onto pre-warmed NZY agar plates. The top agarose was allowed to set, the plates inverted and incubated at 37°C for approximately 6 hours or until pin point size plaques appeared. Sterile nitrocellulose (NC) filters, pre-soaked in 10mM IPTG and then air dried, were laid on the agarose and the plates were incubated at 37°C for an additional 4 hours or until the plaques were approximately 1 to 2 mm in diameter.[42] The orientation of the filter on the plate was marked by making needle holes through the filter and agar with a 22 gauge sterile needle. The filters were removed and washed in 25mM Tris, 28mM NaCl, and 0.001mM K (TBS) for 10 minutes, washed in dd water for 2 minutes, air dried on filter paper, and then baked at 80°C in a hybridization oven. After 5 minutes of baking, the filters were removed; positive and negative controls were dotted onto pre-determined locations and the filters were returned to the 80°C oven for an additional 15 minutes. The filters were briefly submerged in dd water, washed in 10mM acetic acid

for 20 minutes, and then washed aggressively inTBS containing 0.05% Tween (TBST) for 10 minutes. This was followed by the immunological assay.

SMSV-5RT Processing for Screening

The SMSV-5RT library was processed for screening in the following manner.[27] From the stored SMSV-5RT library, random samples were inoculated into 2ml of LB medium containing 50ug/ml of ampicillin into 6ml polystyrene snap top tubes. The tubes were incubated at 37°C with shaking for 8 to 9 hours. From the tubes, isolation streaks were made onto LB 50ug/ml ampicillin plates which were inverted and then incubated overnight at 37°C. These plates constituted the master plates. In the morning, the master plates were cooled at 4°C for 1 hour, colony lifts were made using sterile NC filters, and then transferred colony side up to fresh pre-warmed LB 50ug/ml ampicillin plates. The master plates were incubated for an additional 4 hours at 37°C to re-grow the colonies, and then the plates were sealed with parafilm and stored at 4°C. The transfer plates were incubated for 4 hours at 37°C, after which time the NC filters were removed and placed colony side up on fresh LB 50ug/ml ampicillin plates containing 1mM IPTG. The plates were incubated for an additional 4 hours at 37°C.

The colonies on the NC filters were lysed in the following manner. The filters were placed in a chloroform saturated atmosphere for 15 minutes followed by a 1 minute wash in a solution of 100mM Tris HCl pH 7.8, 150mM NaCl, 5mM $MgCl_2$, 1.5% BSA, 1ug/ml pancreatic DNase I, and 40ug/ml lysozyme (lysis buffer I) and then a 1 minute wash in TBS. The filters were then transferred to fresh lysis buffer I and washed overnight at room temperature. In the morning the filters were removed from the lysis buffer I and washed with three 5 minute washes in TBST. The filters were then washed in dd water for 2 minutes, washed for 20 minutes in 10mM acetic acid, washed for 2 minutes in dd water, and finally washed for 2 minutes in TBS.

Immunological Assay for SMSV-5 Lambda and SMSV-5RT

All wash steps were completed at room temperature in 150mm petri dishes, with 125ml of wash solution, using a Forma Scientific shaker. The filters were blocked with Super Block (Pierce) for 30 minutes, washed for 5 minute in TBS, and then incubated in the primary antibody for 1 hour. The primary antibody was comprised of a panel of polyclonal antibodies, produced in rabbit, against SMSV-5, SMSV-13, and SMSV-17. These polyclonals were purified with column chromatography using *E. coli* antigen absorbed to Cn Br sepharose. A working dilution of 1:500 was used, and TBST containing 0.25% bovine serum albumin (TBST/BSA) was used as the diluent.

The primary antibody was washed off the filters with four 5 minutes washes in TBST. The filters were then incubated for 1 hour in the secondary antibody, goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma) diluted with TBST/BSA to a concentration of 1:30,000. The secondary antibody was washed off the filters with four 5 minute washes in TBST followed by a single 5 minute wash in TBS. The filters were developed in 5-bromo-4-chloro-3-indolyl phosphate nitroblue tetrazolium (BCIP/NBT) (for 10 minutes and then washed with three 2 minute washes in dd water. Positive colonies or plaques were selected, amplified, induced, and the fusion protein purified as follows.

Fusion Protein Purification

Positive colonies were inoculated into 35ml of LB 50ug/ml ampicillin medium and incubated overnight at 37°C with shaking. In the morning, 0.5ml from each culture was removed and stored. The remaining culture was induced by adding IPTG to a concentration of 1mM. The cultures were then incubated with shaking at 37°C for an

additional 4 hours. The cells were centrifuged at 400xg for 5 minutes and the supernatant discarded. The pellets were re-suspended in 300ul of a solution of 50mM Tris pH 8.0, 1mM EDTA, 100mM NaCl (lysis buffer II), and transferred to sterile microcentrifuge tubes on ice. To each reaction tube 1ul of 50mM phenylmethylsulfonyl fluoride (PMSF) and 100ug of lysozyme were added.[28] The reaction tubes were quickly mixed, chilled to 4°C for 5 minutes and then the process repeated for a total of four cycles. The samples were placed on ice. To each sample 400ug of deoxycholic acid was added and the sample was immediately mixed. Every 15 seconds thereafter, the samples were mixed and in the interim they were placed in a 37°C heat block; this 2 step process was continued until the samples were viscous, at which time 426U of DNAse I was added and briefly mixed. The samples were incubated at room temperature for 30 minutes, and then centrifuged at 12,000xg at 4°C for 15 minutes. The supernatant was discarded and the pellets re-suspended in 100ml of dd water. The samples were again centrifuged at 12,000xg, 4°C, for 15 minutes, the supernatant was discarded, and then the pellets were re-suspended in 0.1M Tris pH 8.5 with 2M urea. The samples were triturated, centrifuged at 12,000xg at 4°C for 15 minutes, the supernatant was removed and saved, and the pellets were re-suspended in 100ul of dd water.

Western Blot Analysis

A 15ul aliquot from each sample was added to 15ul of 2x loading buffer and boiled for 5 minutes to denature the protein. Twenty ul per well samples were loaded on a 12% Tris-Glycine gel (Bio Rad) for SDS polyacrylamide gel electrophoresis (SDS PAGE). The gel was run at 100v for 10 minutes and then at 160v for 45 minutes using 5x electrode buffer (5mM Tris, 50mM glycine, and 0.02%SDS)). The proteins were transferred to the NC membrane using a semi-dry apparatus (Semi-Phos, Hoffer Scientific Instruments). A sandwich of blotter paper sheets soaked in protein transfer

buffer, (25mM Tris-Base, 192mM glycine and 0.037% SDS.) the gel, and a NC membrane was placed between the electrodes of the Semi-Phor unit. A current of 10mAmps was applied for 20 minutes to the sandwich, transferring the proteins from the gel to the NC membrane.

The NC membrane was screened with a pool of antibodies (SMSV-5, SMSV-13, SMSV-16, SMSV-17) against calicivirus using the immunological assay previously outlined. Positive samples were re-assayed by western blot, but the lanes of the NC membrane were cut into strips and screened independently against SMSV-13, SMSV-15, SMSV-16, and SMSV-17.

RESULTS

cDNA Library Construction

cDNA was synthesized from RNA and visualized by an ethidium bromide assay (Figure 2). The concentration of cDNA was estimated to be 50ng/ul in a total volume of 10ul.

FIGURE 2

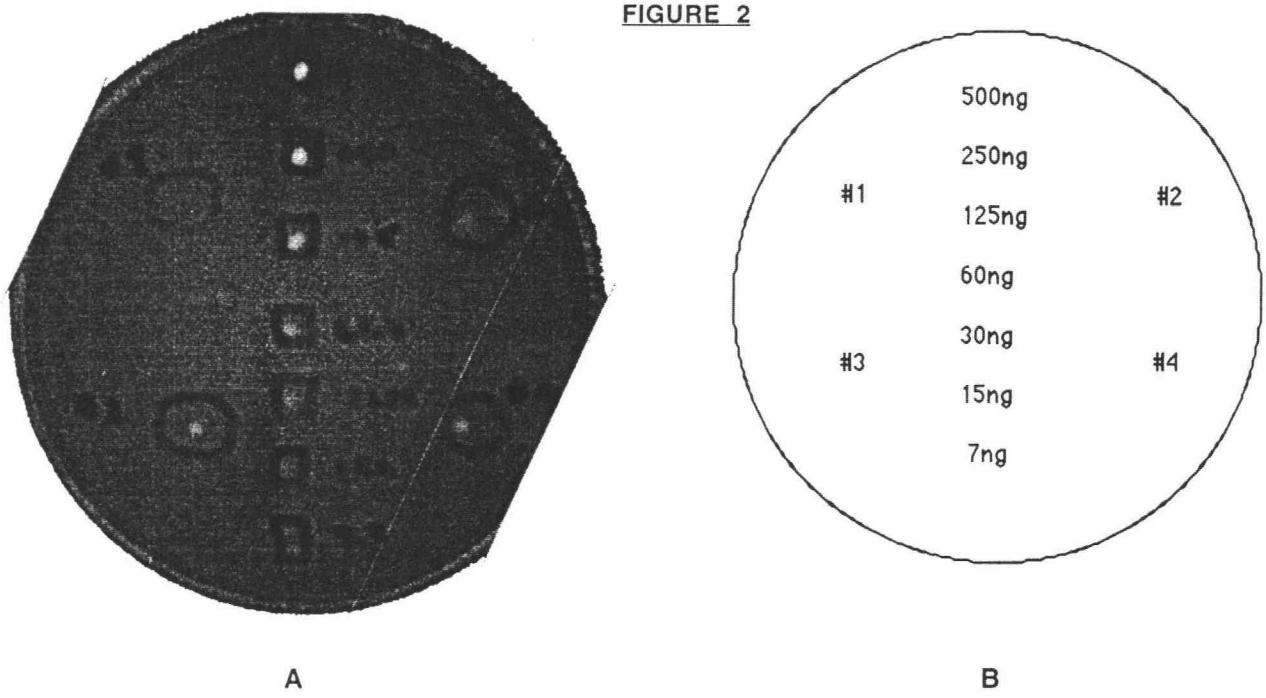

FIGURE 2 cDNA quantification on an ethidium bromide assay plate. Figure 2A shows the ethidium bromide assay plate with standards and samples applied, and Figure 2B is a schematic of Figure 2A. The numbers 1-4 represent the locations where the fractions of cDNA were applied to the agar. The ng values correspond to the locations where known quantities of linear DNA were applied.

The successful cDNA insertions were estimated using blue white color screening (Table 1).

TABLE 1

PLATE	# BLUE PLAQUES	# WHITE PLAQUES
1ul of packaged cDNA	1	24
1ul of a 1:10 dilution of packaged cDNA	2	8
1ul of test DNA	0	40
1ul of vector only	79	0
1ul of wild type lambda	TMTC*	0

TABLE 1 Blue and white color screening assay for packaged cDNA inserts. All plates contained X-gal and IPTG. XL1-Blue was the host cell for all assays, except for the wild type lambda which used the VCS257 host cell (*TMTC too many to count).

Inserts of positive plaques were verified by phagemid excision and visualized on an agarose gel with Et-Br and UV light. Very small inserts are present in samples P-9A and P-11A. Phagemid sample P-1A has a sharp band at approximately 6.1 Kb indicating an insert of approximately 1.6Kb (Figure 3).

FIGURE 3

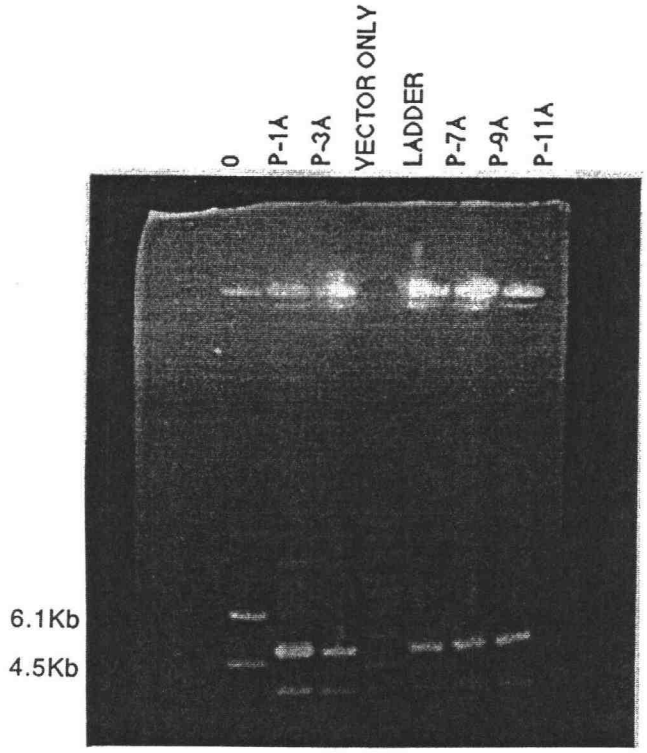

FIGURE 3 Ethidium bromide visualization of phagemid. Agarose gel showing the positive phagemids containing inserts, P-1A, P-3A, and P-9A. The vector only phagemid is 4.5KDa.

cDNA Library Screening and Western Blot Analysis

Positive plaques or colonies were identified by pale purple stippling visible by examination with a dissecting microscope. A positive colony was identified by immunoassay screening and the clone designated 8-SN The clone was isolated, amplified, induced, and the fusion protein assayed via western blot using the same panel of polyclonal antibodies against SMSV-5, SMSV-13,SMSV-15, and SMSV-17 (Figure 4)

FIGURE 4

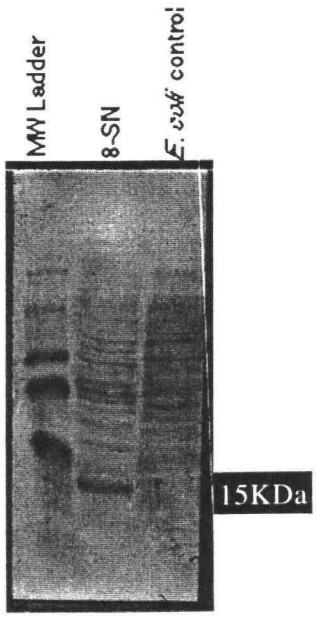

FIGURE 4 Western blot assay of clone 8-SN. Immunological assay consisted on a panel of polyclonal antibodies against SMSV-5, SMSV-13, and SMSV-17. The estimated MW of 8-SN is 15KDa (the B galactosidase protein is approximately 3.5KDa).

The fusion protein of approximately 15KDa is clearly identified from clone 8-NS by four independent western blot assays against SMSV-13, SMSV-15, and SMSV-16, and SMSV-17 (Figure 5).

FIGURE 5

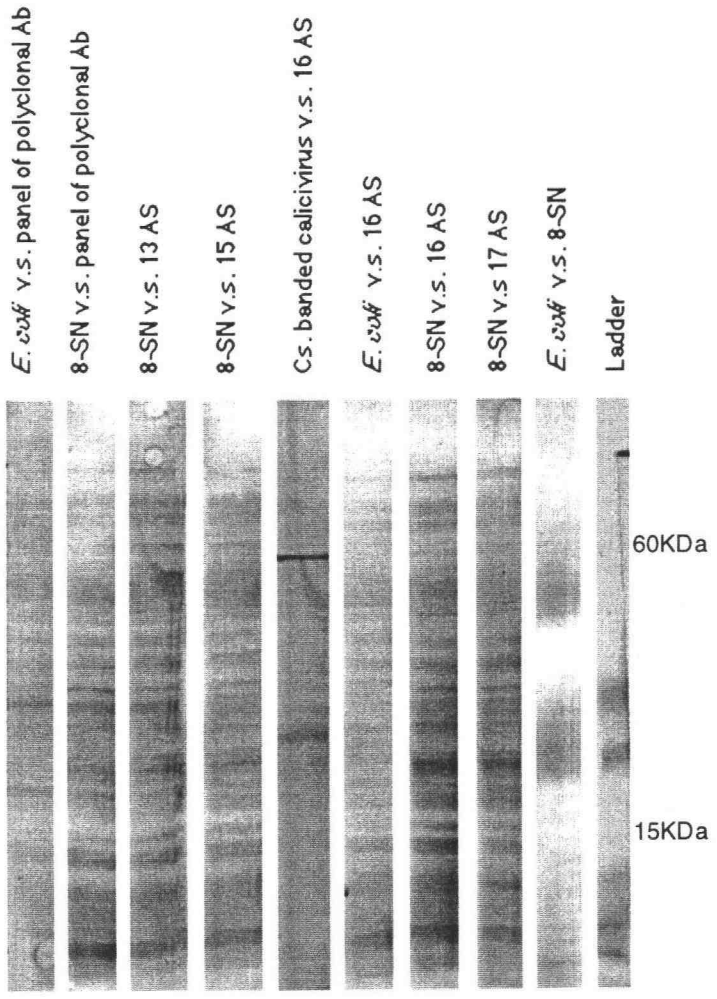

FIGURE 5 Independent western blot assays of the fusion protein 8-SN against antiserum (AS) SMSV-13, SMSV-15 SMSV-16 and SMSV-17. The fusion protein 8-SN reacts positively to independent immunoassay with SMSV-13, SMSV-15, SMSV-16 and SMSV-17 antibodies against calicivirus.

DISCUSSION

Unique cDNA fragments were synthesized from an RNA extract of SMSV-5 infected cells. The cDNA products were tested with an ethidium bromide assay (Figure 2). Since the sample was treated with RNase H during second strand DNA synthesis, the nucleic acid identified on the ethidium bromide assay was indeed cDNA and not RNA. Isolation and examination of the pBK-CMV phagemid from clones containing this ligated cDNA showed an increase in molecular weight when compared to known molecular weight standards and to the known molecular weight of phagemids which do not contain inserts (Figure 3). The most reasonable explanation is the successful synthesis of cDNA and the subsequent ligation of that cDNA into the lambda vector. This conclusion is further supported by demonstrating a disruption of the lac Z gene located within the vector by the blue white color screening results. The cDNA fragments when inserted into the lac Z gene prevent the synthesis of β-galactosidase, a required protein for the production of a blue color in the presence of X-gal. The positive control, test ligation and sample ligation all resulted in the expected frequency of blue to white plaques (Table 1).

The initial screening of both the lambda SMSV-5 and SMSV-5RT libraries resulted in the rapid detection of a positive fusion protein in the SMSV-5RT library. Clones expressing fusion proteins sensitive to the calicivirus antibody panel have not yet been identified in the SMSV-5 lambda library. However, the IPTG induced synthesis of a β-galactosidase fusion protein has been demonstrated with an immuno-assay directed against the β-galactosidase fusion protein in the lambda at varying levels of IPTG (results not shown). These data supports the validity of the expression system and the immuno-assay system. One possible reason for the reduced performance of the lambda library is the use of both cellular and viral RNA to construct the cDNA, although other investigators have successfully used similar RNA extracts lambda expression

system.[8,16,19] Theoretically, by using actively replicating virus in tissue culture as the source of RNA, some of the abundant subgenomic RNA would be incorporated into the SMSV-5 lambda library. In theory, by incorporating the subgenomic RNA into the library, the capsid gene representation would be enhanced. The potential for acquiring a large amount of PK cellular RNA and this competing with lessor amounts of calicivirus RNA was recognized. However, when the lambda vector system was evaluated with its features of directional insertion and high ligation efficiency coupled with a sensitive assay systems, the identification of promising SMSV-5 clones was expected.

A promising clone, designated 8-SN was successfully isolated when from the SMSV-5RT cDNA library. The 8-SN clone when induced does synthesize a polypeptide that is recognized by a panel of polyclonal antibodies against caliciviruses. Once separated by gel electrophoresis this polypeptide was antibody bound using western blot and the polyclonal antibody panel. The positive band was approximately 15kDa. Further, this fusion polypeptide is recognized as having calicivirus epitopes using independent western blot assays for individual polyclonal antibodies not present used in the initial immunoassay (Figure 5). The project objective was to isolate an inducible clone that would produce a fusion protein recognized by a panel of different calicivirus typing serums. The clone 8-SN, meets these criteria for possible application in a simple, rapid, serological assay to detect previous or concurrent calicivirus exposure irrespective of the serotype.

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