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Six serotypes of calicivirus (San Miguel Sea Lion Virus types 4,6,7 and 13, Mink Calicivirus and Reptile Calicivirus) were plaque purified, and two-dimensional gel electrophoresis fingerprints, of the ^{32}P-labeled Ribonuclease T1 digested viral RNA, were performed for each serotype. Fingerprints revealed several oligonucleotides with similar mobilities, and therefore, possibly similar base composition. Each fingerprint revealed what was believed to be Poly A of homogeneous size, and Poly C which migrated a different distance for each serotype. For those viruses studied, there was a direct relationship between plaque size and the length of the Poly C tract. This correlation between plaque size and Poly C migration may serve as a valid indicator for determining pathogenic potential of caliciviruses.

Comparisons of Calicivirus Pathotypes Using Oligonucleotide Patterns from Two-Dimensional Gel Electrophoresis

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COMPARISONS OF CALICIVIRUS PATHOTYPES USING OLIGONUCLEOTIDE PATTERNS, FROM TWO-DIMENSIONAL GEL ELECTROPHORESIS

OBJECTIVES

Genomic differences, as demonstrated by oligonucleotide fingerprints, were examined in order to determine the pathogenic potential of new and unknown calicivirus isolates. The objectives of this research were to plaque purify six serotypes of Calicivirus [San Miguel Sea Lion Virus types 4,6,7 and 13, Mink Calicivirus (MCV 20-3) and Reptile Calicivirus (RCV Cro-1)] and make comparisons of their RNA fingerprints. The group of caliciviruses selected includes isolates exhibiting a wide range of suspected or known pathogenic potential. The single-stranded RNA genome of each serotype was radioactively labeled in vitro with ^{32}P-orthophosphoric acid. The viruses were then purified, digested with Proteinase K and the viral RNA extracted. Fingerprints of the Ribonuclease T1 resistant oligonucleotides of each serotype were constructed and compared using two-dimensional polyacrylamide gel electrophoresis. The Poly C tract and other similarities and differences between the fingerprints of suspected virulent and avirulent viruses were noted as was the relationship between plaque size, Poly C migration and pathogenic potential.

INTRODUCTION

Over the past fourteen years, calicivirus isolations from many animal species have been documented. The host range of caliciviruses is unusually broad, involving aquatic and terrestrial mammals, including: domestic mink(40), cattle(71), dogs(30), cats(32), walrus(75), dolphins(83), snakes and amphibians(69), ocean fish(80), whales(77), Northern fur seals(73), California sea lions(67), Northern elephant seals(84), non-human primates(81) and humans(23).

Members of the family Caliciviridae include Vesicular Exanthema of Swine Virus (VESV) and San miguel Sea Lion Virus (SMSV), which are indistinguishable from each other, and Feline Calicivirus (FCV) (57). Caliciviruses were originally classified as a genus of the family Picornaviridae. However, differences in physico-chemical properties, genome strategy, and the presence of a single major polypeptide have led to their being reclassified as a new virus family, the Caliciviridae (12,17,19,25,33,47,57,58). Caliciviruses are small (35-40 nm.), non-enveloped, and contain a single-stranded RNA genome. The RNA is non-segmented, has a 5' VPg (virion protein covalently linked to a guanine residue), and a poly-adenylated 3' tail. The VPg protein is required for RNA infectivity and remains attached when the RNA is subjected to deproteinization techniques (33,59,61). The nucleocapsid has icosahedral symmetry with cup-shaped indentations; hence the name calicivirus (Latin: calyx). The virus has a buoyant density of 1.36-1.39 g/ml in CsCl, a sedimentation coefficient of 170-183S, and maturation occurs in the cell cytoplasm(50,60,92). Electron micrographs of typical calicivirus-infected cells, reveal condensation of nuclear

material, nuclear membrane alterations and vesicle formation in the cytoplasm. Virions have been shown to accumulate in crystalline arrays and to associate with cisternae and microfibrils. Monolayers of African Green Monkey Kidney (Vero) cells display such cytopathic effects (CPE) as withdrawl of cell processes and dissociation of rounded-up cells from the growth surface(92). Additional characteristics pertaining to the caliciviruses are listed in Table 1.

VESV was first described in 1932, in Buena Park, Orange County, California, and was thought at that time to be foot and mouth disease (FMD)(88). Officials immediately put eradication measures into effect and within ten days the disease was eliminated by slaughter and deep burial of over 18,000 infected and exposed animals (89).

Despite stringent control measures imposed by the Secretary of Agriculture, outbreaks of VESV continued to occur each year, from 1932 to 1936. Experimentally, cattle were unaffected by VESV, but swine developed lesions identical to FMD and some horses developed fever and lingual lesions (88,89). Conversely, Foot and Mouth Disease Virus (FMDV) was known to infect cattle and swine but did not infect horses. Thus, this new agent, VESV, was shown to differ from FMDV (88). Cross-infectivity tests showed that at least four distinct serotypes of VESV were involved (21). Although strict quarantine measures were imposed on infected herds, the disease continued to reappear each spring, even in herds as far as 500 miles away. No satisfactory explanation for the source of virus was found, although most infected herds had been fed raw garbage (43).

Table 1. Characteristics Associated with Caliciviridae

Property	Caliciviridae
Nucleic acid	ss-RNA, non-segmented, MW 2.6-2.8 X 10^6, poly-A 3' terminus; no 5' methylated cap, 5' VPg linked protein.
Morphology	Icosahedral symmetry; cup-shaped indentations; non-enveloped
Size	35-40 nm
Protein	One major capsid polypeptide, MW $71X10^3$; Minor polypeptide, MW $15X10^3$.
Buoyant Density	1.36-1.39 g/ml
Sedimentation Coefficient	170-183 S
Virion Sensitivity	
Heat/$MgCl_2$	Increased lability at 50C/1 M $MgCl_2$
pH	Inactivated at pH values <3
Ether	Stabile
Site of Assembly	Cell cytoplasm

In 1945, a bill was introduced into California legislature that would require the cooking of raw garbage fed to swine, however, the bill failed to pass. Then, in 1952, the disease appeared in a swine herd in Grand Island, Nebraska, and was traced to Cheyenne, Wyoming, where hogs were fed garbage containing uncooked pork scraps that had been unloaded from a train from San Francisco, California (63). VESV soon spread to all swine-producing areas across the United States. The Secretary of Agriculture declared a state of emergency and legislation requiring the cooking of all garbage fed to swine was strictly enforced. The incidence of vesicular exanthema of swine (VES) declined rapidly, with the last reported outbreak in 1956. On October 22, 1959, VES was declared to have been eradicated and designated a foreign (exotic) animal disease (2,43). From 1954 to 1956 this disease cost the federal government over 39 million dollars in direct indemnities and other direct costs (2). Other than an isolated incident in Iceland, where U.S. military personnel on an Air Force base discarded raw pork, from the U.S., into garbage that was subsequently fed to swine, VES outbreaks have never occured outside of the United States. Should a VES outbreak reoccur in the U.S., devastating economic losses would be expected due to decreased productivity and embargos on American meat products.

Mortality from VES, although usually low, did occur in young pigs and was most often associated with abortion and weight loss due to agalactia and eating and breathing interference from vesicle formation around their mouths and nostrils. Morbidity among fattening and breeding animals was very high (21,90).

Clinically VES is indistinguishable from FMD. Initially there is fever and anorexia followed by vesicle formation on the snout, mouth,

feet, and occasionally on the udder and teats. Vesicles erode after a day or two, leaving raw areas. The vesicular fluid contains large quantities of virus, and is extremely important in the spread of infections. Virus-infected feet may become secondarily infected by opportunists, causing lameness, which may lead to a severe reduction in weight gains and overall herd health (88,89). Definitive diagnosis requires isolation and identification of the virus and is necessary for differentiating VESV from the other known viral vesicular diseases (FMD, Vesicular Stomatitis, and Swine Vesicular Disease). Although VESV control and eradication was achieved by quarantine and enforcement of legislation requiring cooking of all garbage fed to swine, the natural reservoir and source of VESV in the garbage was never determined (43).

Beginning in the late 1960's and early 1970's increasing incidents of premature births among California sea lions (Zalophus c. californianus) were reported along the southern California coast. In 1972, while investigating premature whelping on San Miguel Island, California, Smith, et al (67) were able to isolate the first virus obtained from a pinniped species. The isolate was shown to be indistinguishable from VESV, both physico-chemically and in its experimental host range. Mice, rabbits, hamsters and guinea pigs were resistant. Horses were variably susceptible, and swine developed clinical vesicular exanthema (72). This was the first time ever that a virus of marine origin was shown to produce disease in terrestrial mammals. Cross-neutralization tests performed in 1972 on a series of calicivirus isolates from California sea lions and Northern fur seals (Callorhinus ursinus) revealed the presence of two distinct serotypes of virus. These serotypes were designated SMSV-1 and SMSV-2 (72).

With the discovery of SMSV in California sea lions and Northern fur seals and the subsequent finding that SMSV was indistinguishable from VESV, it became accepted by some that these two agents were the same and that both had origins in the ocean (65). Upon finding that marine mammals and fishes could be carriers or possible reservoirs for SMSV, and possibly VESV, an investigation was begun to study the natural history of these ocean viruses and their reservoir hosts.

In 1973, a new SMSV serotype was recovered from an aborted sea lion fetus on San Miguel Island, California, and another was isolated from a Northern fur seal on St. Paul Island, Alaska. These two serotypes were designated SMSV-4 and SMSV-5, respectively (73).

To date, there are 13 recognized serotypes of VESV, the last being reported in 1956, 12 distinct serotypes of SMSV, and three additional serotypes which occur in the ocean but have not received the SMSV designation (Walrus Calicivirus, Bovine Calicivirus BCV-Bos-1, and Cetacean Calicivirus) (11,71,73,75,83).

In 1976, a new serotype (SMSV-7) was isolated from opaleye perch (Girella nigricans) off the California coast (80). This serotype was also isolated from Northern elephant seals (Mirouga angusterostrius) and from a sea lion liver fluke (Zalophatrema spp.). Inoculation studies with SMSV-7 in domestic swine produced a disease clinically indistinguishable from VES (80). In 1977, another serotype, SMSV-6 was isolated from opaleye on San Nicolas Island, California. This serotype had previously been isolated from vesicular lesions on the fore-flippers of sea lion pups on San Miguel Island in 1975 (66,80). A possible transmission cycle for the spread of calicivirus was proposed and studied by Smith, et al (79). Opaleye, feeding on sea lion feces,

become infected with sea lion lung worm larvae which encyst in the gut wall of the fish. The encysted larvae are thought to contain calicivirus that may then be transmitted to pinnipeds or possibly other species when they feed on raw fish.

Although opaleye serve as a reservoir for calicivirus serotypes in southern, warmer, waters, calicivirus isolates found in northern, cooler, waters may be either carried there by migrating marine mammals or exist there in other host species, since no northern reservoir is yet known.

The most recent SMSV serotype to be isolated, SMSV-13, was recovered from an outbreak of vesicular disease at the California Marine Mammal Center (CMMC) in 1984 (11). Antibodies to SMSV-13 were found in 24 out of 29 (24/29) California sea lion pups sampled on San Miguel Island in September, 1984, in 8/66 adult Stellar sea lions from Alaska in May of 1985, and in 3/130 cattle sampled in Oregon in late 1984 (11). Oral lesions, previously unreported in association with SMSV-calicivirus disease, were observed at CMMC as were the more commonly reported flipper lesions. Oral lesions were commonly observed manifestations of VESV in domestic animals.

Of the 12 SMSV serotypes, 8 have been isolated from California sea lions. Serological studies performed on California sea lions have shown not only the presence of SMSV antibodies, but also the presence of type-specific antibodies to several VESV serotypes, indicating that VESV serotypes are still circulating in the Pacific basin (70).

SMSV has now been isolated from several marine species: California sea lions (SMSV-1,2,3,4,6,9,12,13) (66,67,73,80,82), Northern elephant seals (SMSV-7) (80), Northern fur seals (Callorhinus ursinus/SMSV-

1,5,6,8,10,11,12) (72,73,80,82), Opaleye fish (SMSV-6,7) (80), walrus (Odobenus rosmarus/WCV) (75) and Atlantic bottlenosed dolphin (Tursiops truncatus Cetacean Calicivirus CCV Tur-1) (83).

Additional caliciviruses have been isolated from domestic mink (MCV) (40), domestic cattle (Bovine Calicivirus BCV Bos-1)(71), reptiles and amphibians (RCV Cro-1) (69), domestic dogs and Pygmy Chimpanzee (Pan paniscus/Primate Calicivirus PCV Pan-1) (30,81).

In addition several animal species have not yielded virus isolates, but have been tested positive for specific serum-neutralizing antibodies to VESV and SMSV. These are the Gray whales (Eschrichtius gibbosus),Sei whales (Balaenoptera borealis), Sperm whales (Physter catodon), feral swine, donkeys, sheep, buffalo, and foxes. (1,54,68,70,77).

In addition to the Pygmy Chimpanzee, the serotype PCV Pan-1 has been isolated from the following species in a zoological collection: spider monkey (Ateles fusciceps), douc langer (Pygathrix nemaeus), silver leaf langur (Presbytis cristata), and lowland gorilla (Gorilla gorilla) (76,78,81).

Several researchers have reported on a calicivirus-like agent called the Norwalk agent, associated with infant diarrhea and epidemic gastroenteritis of adults (20,25,56). Although the Norwalk agent has not been isolated in vitro, there is a single report on the isolation and propagation of a human enteric calicivirus (23).

The relative ease with which caliciviruses from the ocean can be propagated in primate cells, and the wide range of host species from which they have been isolated, suggests that caliciviruses may infect human beings. Prior to 1985, antibodies to SMSV-4 and SMSV-5 had been reported in several researchers, but no illness was reported (74,81).

Serotypes of calicivirus are determined using serum cross-neutralization tests. Although VESV and SMSV do not cross-neutralize, they do show some measure of antigenic relatedness using immunodiffusion, immunoelectron microscopy, and radioimmune precipitation tests. These two virus groups are indistinguishable and the isolates designated SMSV are by existing criteria, simply new serotypes of VESV, which was declared eradicated from domestic swine in 1959 (17,19,57,65,67,84). FCV isolates cross react with each other and belong to a single serotype (16,42,51). FCV is ubiquitous among cat populations throughout the world, and inner-strain variation in antigenicity, pathogenicity, and tissue tropism have been reported (16,51).

Many methods are available for differentiating one virus from another, including; neutralization testing, plaquing characteristics, immunoelectron microscopy, complement fixation, hemagglutination activity, infectivity studies, and cytopathic effects. Several calicivirus isolates, including all SMSV serotypes, have been plaque purified using the techniques described by Dulbecco and Vogt (28). Plaque types observed with VESV, SMSV, and FCV, range from large, circular, plaques with diameters of 4 to 8 mm, to minute, irregular, plaques having diameters less than 1.5 mm (45,46,51,52,).

McClain, et al (46) performed infectivity studies of plaque-purified populations and found that large plaque variants were highly virulent, while minute plaque variants were essentially avirulent. Virus isolated from lymph nodes of animals that had recovered from all clinical signs were shown to be of the minute plaque type, suggesting

that minute variants may be the dominant form in persistantly infected animals (46).

Multicyclic growth studies have shown that minute plaque variants were able to adsorb to cells and multiply to higher titers than large plaque variants. Large plaque variants were released earlier and more completely from host cells than were minute plaque variants. Large plaque variants flourished over a wide pH range in cell cultures, while minute plaque variants were drastically inhibited at low pH (46). Minute plaque variants were also shown to have less complex energy requirements; they were capable of replicating in energy and nitrogen starved cells (45,46). Studies by Walen, et al (91) showed that mutation, from one plaque type to another, occured in cell culture and in infected pigs.

Of the methods available for differentiating caliciviruses, RNA sequencing of each serotype is the most sensitive. While direct sequencing techniques for RNA genomes are available, complete sequencing requires efficient methods of oligonucleotide separation and is therefore very time consuming and expensive. The following methods have been used for this: adsorption, ion exchange, gel filtration, and electrophoresis. (26,27,39,86).

DeWachter and Fiers (26,27) reported a method of two-dimensional polyacrylamide gel electrophoresis, whereby complex mixtures of RNA molecules were separated and recovered with little or no loss. Oligonucleotides and mononucleotides were successfully separated using this method as follows: RNA, labeled with ^{32}P-orthophosphate, was partially digested with ribonuclease T1 (RNase T1). The first dimension of separation was carried out on 10% polyacrylamide gels, with a low pH,

in the presence of a high urea concentration. Complexes consisting of smaller fragments dissociated, and the distance that the components migrated depended on their size and their base composition (26,27). At low pH the net charge per residue was different for each base. Therefore, electrophoresis resulted in migration which depended more on the base composition than on the size of the oligonucleotides. Effects of secondary and tertiary structure were lost when electrophoretic temperatures and/or concentrations of denaturing agents (e.g. urea) were increased (27).

Electrophoresis in the second dimension was carried out on gels with twice the polyacrylamide concentration as the first gel, no urea, and at a higher pH. Oligonucleotide migration in this dimension was a function of molecular weight and/or secondary structure which tended to reform at this pH (26,27,49,86). The larger the oligonucleotide, the more slowly it migrated from the origin of the second dimension. The oligonucleotides generated were used to show the overlaps necessary for the reconstitution of the complete RNA sequence.

Two-dimensional gels can be autoradiographed for rapid visual comparison. Oligonucleotide fingerprints remain a very sensitive method of detecting some mutational changes, although differences in fingerprints do not necessarily reflect differences in gene products (27,39). Rapid analysis of an RNA genome by fingerprinting has proven to be a very useful and accurate method for assessing molecular aspects within several virus families (34,39,49), including the Caliciviridae (52).

The genomic fingerprints of two caliciviruses, SMSV-5 and SMSV-8 (which exhibited both minute and large plaque characteristics), have

been compared, and each plaque variant for each serotype was unique. A polyadenylated (Poly A) tract was identified for each serotype, as was a cytosine rich (Poly C) tract with migration characteristics that were consistent between plaque sizes, irrespective of serotype. Minute plaque variants exhibited long Poly C tracts on the fingerprints, indicating short Poly C sequences in their genomes, whereas large plaque variants displayed short Poly C tracts, indicating long Poly C sequences in their genomes (52).

In order to determine the validity of using the plaque size and the length of Poly C tract as possible indicators of cytolytic activity and pathogenicity, we examined a group of calicivirus isolates with known levels of pathogenicity for swine. Caliciviruses SMSV-4,6,7 and 13, Mink Calicivirus (MCV 20-3) and Reptile Calicivirus (RCV Cro-1) were selected. Infectivity studies (Berry unpublished), indicate that this group of viruses exhibit pathogenic potential in swine ranging from seroconversion only (RCV and MCV), to classical clinical vesicular exanthema (SMSV-13). Two-dimensional polyacrylamide gel electrophoresis of these serotypes was performed to compare the Poly C tract lengths and other oligonucleotide differences between virulent and avirulent strains.

Perceivable differences in the two-dimensional fingerprints of caliciviruses, such as the migration distance of the Poly C, may be useful for determining pathogenic characteristics of viral isolates, especially when new outbreaks of caliciviral disease occur. Two-dimensional fingerprinting of the caliciviruses should be a valuable tool for demonstrating genomic differences that may reflect cell associatedness, pathogenicity, and other possible differences.

MATERIALS AND METHODS

Virus Source

The calicivirus prototypes used were isolated from several animal species as follows: SMSV-4,6 and 13 were isolated from California sea lions on San Miguel Island, California, in 1973, 1975, and at the California Marine Mammal Center (CMMC), San Francisco, California, in 1984, respectively (73,66,11). SMSV-7 was isolated from opaleye perch (Girella nigricans) off of San Nicolas Island, California, in 1977 (80). Reptile Calicivirus (RCV Cro-1) was isolated from 4 poikilothermic species in a San Diego, California, zoological collection in 1978 and Mink Calicivirus (MCV 20-3) was isolated from domestic mink on a ranch in Idaho in 1977 (69,40). All of the serotypes were previously passaged in Vero cells, except for RCV which was previously passaged in Porcine Kidney (PK) cells.

Virus Titration

Stock virus concentrations were determined by computing the 50% endpoints (Tissue Culture Infectious Dose; $TCID_{50}$) as described by Reed and Muench (55). Accumulated values for the total number of tissue culture wells infected in a 96-well microtiter plate were calculated by adding in the directions indicated by the arrows in columns c and d of Table 2. The accumulated infectivity ratio (column g) represents the accumulated number of infected monolayer wells (column e) over the accumulated total number; for example, at the 10^{-6} dilution, 8 of 11

Table 2. Example of Endpoint Titration

Virus Dilution (a)	Infectivity Ratio (b)	Infected Wells (CPE) (c)	Uninfected Wells (No CPE) (d)	Accumulated Values: Total Infected (e)	Total Uninfected (f)	Ratio (g)	% (h)
10^{-1}	8/8	8	0	48	0	48/48	100
10^{-2}	8/8	8	0	40	0	40/40	100
10^{-3}	8/8	8	0	32	0	32/32	100
10^{-4}	8/8	8	0	24	0	24/24	100
10^{-5}	8/8	8	0	16	0	16/16	100
10^{-6}	5/8	5	3	8	3	8/11	73
10^{-7}	2/8	2	6	3	9	3/12	25
10^{-8}	1/8	1	7	1	16	1/17	6
10^{-9}	0/8	0	8	0	24	0/24	0
Control (Vero cells without virus)							

monolayers were infected. In Table 2, the infectivity at the 10^{-6} dilution is above 50%; that in the next lower dilution, 10^{-7}, is below 50%. Therefore, the 50% endpoint lies somewhere between the 10^{-6} and 10^{-7} dilutions of the inoculated virus. The 50% infectious dose is calculated in column h as follows:

$$\frac{\text{\% infectivity at dil. above 50\% - 50\%}}{\text{\%infec. at dil. above 50\% - \% infec. at dil. below 50\%}} =$$

$$= \text{proportionate distance} = \frac{73-50}{73-25} = 0.479$$

It is necessary to correct the proportionate distance by the dilution factor used, which is the logarithm of the dilution steps employed. In the case of serial ten-fold dilutions, the factor is 1 (log 10=1). The $TCID_{50}$ equals the negative log of the dilution above 50% infectivity, plus the proportionate distance factor. In the example from Table 2, the following value is obtained:

$$TCID_{50}/0.025 \text{ ml} = -\log \text{ of } 10^{-6.479} \text{ or } 3.01X10^{6}$$

Virus titers, expressed as $TCID_{50}$ per ml, were calculated as described above, using 96-well microtiter plates (Corning) containing 100 ul Vero cells ($5X10^{7}$ cells/ml), 25 ul of Eagle's Minimum Essential Media with Earle salts (MEM, Gibco Laboratories), and 25 ul of each plaque purified stock virus (log-diluted 10^{-1} thru 10^{-8}). MEM was supplemented with 7.5% FBS (Hyclone Laboratories), 200 U penicillin, 100 ug streptomycin, 20 ug gentamicin, and 292 ug L-glutamine per ml. Plates were incubated at 37 C in an atmosphere of 95% air and 5% CO_2, and read at 72-96 hrs.

Once virus titers were known, a 100 $TCID_{50}/0.025$ ml was obtained for each virus in order to perform the cross-neutralization tests.

Virus dilution controls (back titrations) (Table 3) were performed to ensure that each 100 $TCID_{50}$ was accurate. The virus dilutions (100 $TCID_{50}$/0.025 ml) were serially diluted with MEM. One 96-well microtiter tray was used as follows: To each of 4 replicate wells 25 ul of virus dilution was added, along with 0.1 ml of Vero cells (5 X 10^7 cells/ml). Plates were incubated at 37 C (5% CO_2, 95% air), and read at 72 hours post-inoculation. Virus stocks were then adjusted to 100 $TCID_{50}$/0.025 ml for the cross-neutralization tests.

Cross-Neutralization Tests

Cross-neutralization tests, using stock antisera, were performed against each isolate to confirm their identity (Table 4). One stock virus was tested against three stock antiseras on each 96-well plate, using serum dilutions of 1:5 thru 1:640. The first 4 wells each received 50 ul of a 1:5 dilution of known stock antisera (heat-inactivated at 57 C for 1 hour). The remaining wells received 25 ul of MEM. A 25 ul diluter was then used to dilute the antisera two-fold across the 96-well tray. Each well then received 25 ul of virus stock (100 $TCID_{50}$). The virus-serum mixture was allowed to incubate at room temperature (23 C) for 60 minutes. Vero cells (100 ul/well; 5X10^7 cells/ml) were then added to each well. The plates were incubated at 37 C in 5% CO_2 and 95% air and then read at 72 hours post-inoculation. Antibody titer was calculated as the 50% endpoint. Cell culture and virus dilution (back titration) controls were run at the same time. Results of the cross-neutralization tests are shown in Table 4.

Table 3. Virus Dilution Control (Back Titration)

10^2	10^1	10^0	10^{-1}	10^2	10^1	10^0	10^{-1}	10^2	10^1	10^0	10^{-1}
+	÷	+	-	+	+	-	-	+	÷	+	-
+	÷	-	-	+	÷	-	-	÷	+	÷	-
+	+	+	-	+	+	-	-	+	+	+	-
+	+	-	-	+	+	+	-	+	+	+	-
100 $TCID_{50}$/.025 ml				47 $TCID_{50}$/ .025 ml				316 $TCID_{50}$/.025 ml			

(÷) Positive CPE

(-) Negative CPE

$TCID_{50}$ calculated by the method of Reed and Muench (55).

Each dilution was done in 4 replicates. Each well received 25 ul of the indicated dilutions plus 0.1 ml of Vero cells. Trays were read between 72 and 96 hours post-inoculation. $TCID_{50}$'s which read at 100 were used as is, and those below or above $TCID_{50}$ of 100 were concentrated or diluted respectively to reach 100 $TCID_{50}$/ .025 ml.

Table 4. Cross-Neutralization Test

Stock Virus						Stock Antisera
SMSV-4	SMSV-6	SMSV-7	SMSV-13	MCV	RCV	
1:320	-	-	-	-	-	Anti-SMSV-4
-	1:160	-	-	-	-	SMSV-6
-	-	1:320	-	-	-	SMSV-7
-	-	-	1:640	-	-	SMSV-13
-	-	-	-	1:80	-	MCV
-	-	-	-	-	1:80	RCV

(-) No protection at antisera dilutions of 1:5 or greater.

Polyclonal Antibody Production

After confirming the serotype of each calicivirus, stock virus was clarified by centrifugation at 2500 xg for 15 minutes (Beckman IEC Centra 4) in 1.5 ml Eppendorf centrifuge tubes. New Zealand white rabbits were used to produce antibodies as follows: Each rabbit was bled (10 ml via cardiac bleeding) and then injected with 1.0 ml of clarified stock virus intramuscularly (I.M.). On day 15 each rabbit was boosted with an additional 1.0 ml I.M. On days 30 and 45 rabbits were bled (10 ml) and injected with 0.5 ml given intravenously (I.V.) in each ear. Sera were tested for neutralizing antibodies on day 45, and all rabbits, except the one injected with SMSV-13, were positive. The rabbit injected with SMSV-13 was again given a booster injection I.V. in the ear, but failed to produce antibody levels above a 1:5 dilution.

On day 52 all rabbits were anesthesized with ketamine and exsanguinated by cardiac puncture.

Serum-neutralization (SN) tests were performed to determine the level of serum antibody. A typical 96-well plate for SN tests is shown in Table 5. Stocks of three viruses were tested on each plate, using serum dilutions of 1:5 thru 1:640. The first 4 wells each received 0.05 ml of a 1:5 known stock sera (previously heat-inactivated at 57 C for 1 hour). To the remaining wells, 0.025 ml of MEM, without (FBS), was added. A 25 ul microdiluter was then used to dilute the sera two-fold across the 96 well tray (1:5 thru 1:640). To each well, 25 ul of appropriate virus stock (100 $TCID_{50}$) was added. The virus-serum mixture was allowed to incubate at room temperature (23 C) for 60 minutes. Vero cells (0.1 ml/well; $5X10^7$ cells/ml) were then added to each well. The plates were incubated at 37 C in 5% CO_2 and 95% air for 72 hours. The

Table 5. Serum-Neutralization Plate (Titration of Positive Sera)

TRAY 1			DILUTION	TRAY 2		
SMSV-4	SMSV-6	SMSV-7		SMSV-13	MCV 20-3	RCV Cro-1
- - - -	- - - -	- - - -	1:5*	- - + +	- - - -	- - - -
- - - -	- - - -	- - - -	1:10	+ + + +	- - - -	- - - -
- - - -	- - - -	- - - -	1:20	+ + + +	- - - -	- - - -
- - - -	- - - -	- - - -	1:40	+ + + +	- - - -	- - - -
- - - -	+ + - -	- - - -	1:80	+ + + +	- - - -	+ - + -
- + + -	+ + + +	- - - -	1:160	+ + + +	+ - - +	+ + - +
+ + + +	+ + + +	- - + -	1:320	+ + + +	+ + + +	+ + + +
+ + + +	+ + + +	+ - - +	1:640	+ + + +	+ + + +	+ + + +

(-) Cells protected by antisera against corressponding virus.

(+) Cells not protected by antisera (Positive CPE).

Antibody titer was calculated as the highest dilution where 50% of the 4 replicate wells were protected (50% endpoint).

* Each dilution was performed in quadruplicate.

antibody titer was calculated as the 50% endpoint. Cell culture and virus dilution (back titration) controls were run at the same time.

Plaque Assay

Stocks of SMSV-4,6,7 and 13, Mink Calicivirus (MCV 20-3) and Reptile Calicivirus (RCV Cro-1), which had been previously purified by three plaque passages, were single plaque purified through three additional passages using a modification of the plaque technique of Dulbecco and Vogt (28). RCV was adapted to Vero cells, from PK-15 cells, using six blind passages on roller tubes containing Vero cell monolayers. For plaque assays, monolayers of Vero cells were cultured in 6-well trays (35 mm wells) at 37 C in an atmosphere of 5% CO_2 and 95% air. MEM was used as the growth media.

Log dilutions (10^{-4} thru 10^{-8}) of all stock viruses were made, using MEM without FBS, as diluent. The growth medium was removed from the Vero monolayers, and 0.5 ml of the appropriate virus dilution was added to one well each, and 0.5 ml of MEM was added to one well for mock-infection. The virus was adsorbed to the Vero cells for one hour at 37 C in 5% CO_2, 95% air, then the monolayers were rinsed twice with 2 ml of MEM to remove unadsorbed virus.

All wells were overlayed with 2.0 ml of medium containing 3.0% noble agar (Difco) mixed 1:1 with 2X MEM, 2% FBS, 10 ul $NaHCO_3$, 150 ug L-cysteine, and 20 ul L-glutamine per ml. The agar overlay was allowed to solidify and then the trays were incubated at 37 C in 5% CO_2 and 95% air until plaques became visible (24-96 hrs.).

Monolayers with plaques were stained for 2 hours at 37 C with a sterile solution of the vital stain neutral red (1:25 in sterile

water). Staining was needed to help visualize minute plaques. A pasteur pipette, containing 0.2 ml of MEM, was used to remove an agar plug from above a single, well isolated plaque. This was repeated for each serotype. The agar plugs were macerated in small snap cap tubes and then inoculated onto 24-hour-old monolayers of Vero cells in 15 ml roller tubes. Virus was allowed to adsorb to the Vero cells for 1 hour at 37 C, then removed, and cells were re-fed with 1.5 ml of MEM containing 2% FBS as described above. The inoculated tubes were incubated at 37 C, on roller drums turning at 0.3 rpm, until 75-100% cytopathic effect (CPE) was observed, then frozen at -70 C, thawed, and clarified at 1700 xg (IEC Model UV) for 20 minutes. Supernatant was removed and stored at -70 C for furthur plaque passaging.

Representative plaques for each serotype were stained and prepared for photographing as follows: Cells were fixed with a 10% formalin solution for 30 min. Then the agar overlays were removed with a small spatula. Care was necessary during this step to avoid scratching the fixed monolayers. Crystal violet (1 ml of 0.5% solution in methanol) was then added to furthur enhance plaque appearance (15 sec). Crystal violet was rinsed from the trays with running tap water. Trays were allowed to air dry, and were then photographed using Kodak Technical Pan film in a Nikon F3 camera, equipped with a 55 mm Macro Lens.

Virus Amplification

Flasks (Corning 75 cm2) containing confluent monolayers of Vero cells were inoculated with 0.1 ml of each plaque-purified stock virus. When 75-100% CPE (4+ CPE) was observed (18-24 hrs.), each flask was freeze-thawed (-70 C) once to enhance virus release. The contents were

centrifuged (IEC Model UV) at 1700 xg for 20 minutes, and stored in 4 ml vials at -70 C.

In vivo ^{32}P-labeling of Calicivirus RNA

Vero cells were grown to confluency (24-48 hrs) in 850 cm^2 roller bottles (Corning), using 100 ml MEM with 10% FBS. To ensure a large quantity of ^{32}P-labeled viral RNA, two roller bottles were inoculated for each serotype to be fingerprinted as follows: stock virus (0.75 ml) was diluted with 10 ml MEM, without FBS. Five ml of diluted stock virus was inoculated onto the monolayers in each roller bottle and allowed to adsorb for 2 hours at 37 C, while rotating at 0.3 rpm. The inoculum was then poured off, and cells were rinsed once with 25 ml of phosphate-free MEM (PFMEM). PFMEM was prepared as follows: 0.2 grams anhydrous $CaCl_2$, 0.4 grams KCL, 0.2 grams $MgSO_4$ $7H_2O$, 6.8 grams NaCl, 1.0 gram D-glucose, 0.01 gram phenol red, 20 ml of 50X MEM Essential Amino Acids, 10 ml 100X MEM Vitamins, 10 ml L-glutamine, 10 ml Penicillin-Streptomycin solution, and 2 ml gentamicin (Antibiotics were in same concentration as MEM prepared earlier). The mixture was brought to a total volume of 1 liter with dd-H_2O, with 1% $NaHCO_3$. PFMEM was then filtered through a 0.2 u filter to remove contaminants. Roller bottles were refed with 25 ml PFMEM with 8% dialyzed FBS (Gibco). Actinomycin D, which inhibits DNA-dependent RNA synthesis, was added at a concentration of 2 ug/ml of media (50 ug/roller bottle). The ^{32}P-orthophosphate (New England Nuclear) label was added to the roller bottles at a rate of 60 uCi/ml (1.5 mCi/bottle). Roller bottles were then incubated at 37 C, and 0.3 rpm, until CPE reached 4+ (18-36 hrs). MCV ($1.5X10^6$ $TCID_{50}$/ml) required 48 hours before 4+ CPE was observed.

^{32}P-labeled Virus Purification

The procedure for purifying the virus was modified from the method of Oglesby, et al(50), Schaffer and Soergel (60), and Poet (52). The roller bottle supernatant, containing ^{32}P-labeled virus, was pipetted into 50 ml, screw-cap, centrifuge tubes (Falcon). Roller bottles were rinsed with 10 ml of TNE buffer (15 mM TRIS, 1M NaCl, 1 mM EDTA, pH 7.5), which was then combined with the tissue culture media. The suspension was then freeze-thawed 3 times to release virus from intact cells (-70 C; 37 C water bath), and then vortexed, transfered to fresh 50 ml centrifuge tubes, and centrifuged at 1700 xg for 20 minutes (IEC Model UV) to remove large cell debris. The supernatant was poured into sterile, siliconized (Prosil, Sigma), polyallomer ultracentrifuge tubes, and then centrifuged at 80,000 xg, at 4 C for 2 hours to pellet the virus (SW-27 rotor; Beckman L8-70 ultracentrifuge). The pelleted virus was resuspended in 300 ul TNE buffer (pH 7.5).

The resuspended virus was then layered onto a 1.38 g/ml CsCl solution in a siliconized Beckman ultra-clear (13 X 51 mm) centrifuge tube, and centrifuged at 4 C, at 150,000 xg, for 18 hours in a Beckman L8-70 ultra-centrifuge using an SW 50.1 rotor.

The light-scattering band, containing the purified virus, was removed by side-puncture using a 23 gauge needle with a 1 ml syringe. The virus was placed into another siliconized ultra-clear centrifuge tube, diluted 1:5 with TNE buffer (pH 7.5), and centrifuged at 150,000 xg and 4 C for 2 hours to remove CsCl and to pellet the virus. The pellet of purified virus was then resuspended in 400 ul of TE buffer (15mM TRIS, 1 mM EDTA, pH 8.0).

Virus preparations were checked for purity by electron microscopy, using the method of Skilling, et al (64), by placing a 5 ul sample on Parafilm and then floating a 300 mesh, Formvar coated, carbon stabilized grid face downward on the virus sample for 30 seconds. The grid was blotted dry, then floated on a drop of distilled, sterile, water for 5 seconds, blotted dry, and floated on a drop of 1.5% phosphotungstic acid in water for 30 seconds. The grid was blotted dry and examined with a Philips, model 300, Transmission Electron Microscope at 80 KV and 50,000 X magnification.

Protein Digestion of Virus

Twenty microliters of 10% Sarkosyl (TE, pH 8.0) was added to the purified virus-TE (TRIS-EDTA) suspension contained in the ultra clear centrifuge tube to give a 0.5% Sarkosyl concentration. Eight microliters of proteinase K (stock 5 mg/ml H_2O, Bethesda Research Laboratories) was then added to give a concentration of 100 ug/ml. The centrifuge tube was covered with Parafilm and allowed to react for 2 hours at 65 C.

Viral RNA Extraction

The proteinase K digest was transferred to a siliconized 1.5 ml Eppendorf centrifuge tube for phenol-chloroform extraction. The phenol solution was prepared as follows: 8-hydroxyquinoline (antioxidant-ribonuclease inhibitor, Mallinckrodt) was added to 150 ml of phenol to give a 0.1% concentration; 150 ml of 1M TRIS buffer (pH 8.0) was added to the phenol and shaken for 10 minutes. The mixture was allowed to separate overnight at 4 C. After separation, the upper layer was discarded. TE buffer (100 ml) was added, shaken for 10 minutes, allowed

to separate at 4 C, and the upper layer discarded. Another 100 ml of TE buffer was added, mixed, and allowed to separate. The solution was placed in a dark bottle and stored at 4 C until needed (bottom layer).

The RNA was extracted twice by adding 500 ul of the buffer-saturated phenol to the sample. The samples were shaken and then centrifuged at 10,000 xg (Beckman IEC Centra 4) for 1.5 minutes. The bottom (phenol) layer was discarded each time. The sample was then treated with 500 ul of a 24:1 chloroform-isoamylalcohol solution, shaken, centrifuged at 10,000 xg for 30 seconds, and the bottom layer discarded (removing any remaining phenol).

The sample RNA was precipitated by adding 100 ug of transfer RNA (t-RNA) (2mg/ml H_2O stock t-RNA, Bethesda Research Laboratories), and 80 ul of 5M sodium acetate, to give a final salt concentration of 0.4M. One ml of cold (-20 C) 95% ethanol was added to the mixture. The solution was shaken and placed at -70 C for at least 30 minutes. The RNA was pelleted by centrifugation at 10,000 xg for 15 minutes. The supernatant was discarded and the pellet dried.

RNase T1 Digestion of ^{32}P-labeled RNA

The procedure was modified from the methods of Clewley, et al (22), Frisby, et al (34), and Poet (52). The RNA pellet was resuspended in 20 ul of 0.01 M TRIS (pH 7.6), and 2 ul of ribonuclease T1 (10 units/ul stock RNase T1, Bethesda Research Laboratories). The RNA was allowed to digest for 1 hour at 37 C. The reaction was stopped by adding solid urea to a concentration of 6M. Ten ul of loading buffer (6M urea, 50% sucrose, 0.2% xylene cyanol, and 0.2% bromophenol blue) were added to

the sample and the mixture was heated to 65 C for 3 minutes immediately prior to loading the sample onto the first dimension gel.

Two-dimensional PAGE of ^{32}P-labeled Viral RNA

The procedure was modified from the methods of DeWachter and Fiers(26,27), Clewley, et al (22), Frisby, et al (34), and Poet (52).

The first dimension gel was made by combining 40% acrylamide/1.3% bis-acrylamide stock solution, 250 mM citric acid buffer (pH 3.5), urea, and double-distilled water to give a gel composition of 10% acrylamide/ 0.33% bis-acrylamide. The solution was allowed to come to room temperature (23-25 C), and was then degassed under vacuum with gentle swirling for 8 to 10 minutes (Degassing promotes polymerization). Catalysts were added to polymerize the gel: 10% ascorbic acid (0.94 ul/ml of gel), 2.5% $FeSO_4$ $7H_2O$ (0.94 ul/ml of gel), and 30% H_2O_2 (0.094 ul/ml of gel). The gel was poured immediately, using an LKB 2001 Double Length Verticle Electrophoresis Unit (18 cm X 32 cm X 1.5 mm). All gels were allowed to polymerize at room temperature for at least 90 minutes prior to electrophoresis.

The RNA sample (20-30 ul) was loaded onto the gel. Electrophoresis was carried out at 400V (constant voltage), 20-40 mA and 23 C, until the bromophenol blue dye (green at pH 3.5) had migrated 19 cm (4 to 7 hours). The electrophoresis buffer was 25 mM citric acid, pH 3.5 and 6M urea in dd H_2O.

Two samples of viral RNA were run at the same time in the first dimension gel to minimize the expense of urea and acrylamide. All first dimension strips (2 cm X 28 cm) were run immediately in the second

dimension strips (2 cm X 28 cm) were run immediately in the second dimension, although Poet (52) reported no detectable differences in two-dimensional patterns from strips stored at 4 C for up to 3 days.

After the first dimension electrophoresis, the glass plates were separated and the strip containing the oligonucleotides was cut free of the remaining gel. The glass plate was inverted over a second dimension plate (30 cm X 40 cm X 1.5 mm) and the gel was placed 6 cm above, and parallel to, the bottom edge. The second dimension gel mold was then sealed with an agarose mixture (0.75%, in 100 mM TRIS-borate and 2.5 mM EDTA, pH 8.3), which had previously been autoclaved and held at 47 C.

The second dimension gel was made by combining 40% acrylamide/1.3% bis-acrylamide stock solution, 1M TRIS-borate (pH 8.3), 100 mM EDTA (pH 8.3), and double-distilled water to give a final gel composition of 20% acrylamide, 0.65% bis-acrylamide, 100 mM TRIS-borate, and 2.5 mM EDTA.

The gel solution was allowed to come to room temperature and degassed for 8 to 10 minutes prior to adding freshly prepared 10% ammonium persulfate and TEMED (N,N,N',N'-Tetramethylethylenediamine, Sigma) to catalyze the polymerization. The gel solution was poured immediately after adding the catalysts, and allowed to polymerize for two hours at room temperature. The second dimension gel buffer contained 100 mM TRIS-borate and 2.5 mM EDTA (pH 8.3). Electrophoresis was performed using an LKB 2001 Power Source, set at 600V (constant voltage), with 120 mA (dropping to approximately 36 mA at finish), at 4 C. The gel was allowed to run until the bromophenol blue dye (blue at pH 8.3) migrated 19 cm (12 to 19 hours).

Autoradiography

Once the second dimension electrophoresis was complete, the glass plates were separated, the gel blotted dry, allowed to come to room temperature, and then wrapped in Saran Wrap. Allowing the gel to come to room temperature and dry prevents condensation from appearing on the film. X-ray film (Kodak X-Omat, 14 X 17 inches) was placed over the gel, covered with another glass plate, and wrapped with foil and paper to block light. The gel was placed in the dark at 15 C, film side down, for exposure (12 to 120 hours, depending on the amount of radioactivity in the gel) After exposure, the film was developed using Kodak GBX X-ray developer.

RESULTS

Virus Titers

Virus titers were as follows: SMSV-4, $1.86X10^8$ $TCID_{50}$/ml; SMSV-6, $8.6X10^7$ $TCID_{50}$/ml; SMSV-7, $2.56X10^9$ $TCID_{50}$/ml; SMSV-13, $1.03X10^7$ $TCID_{50}$/ml; RCV, $1.28X10^8$ $TCID_{50}$/ml and MCV, $1.5X10^6$ $TCID_{50}$/ml.

Cross-Neutralization

Results of the cross-neutralization tests are shown in Table 4. Stock virus was neutralized by corresponding stock antisera at titers ranging from 1:80 to 1:640. However, non-corresponding antisera provided no cross-neutralization at dilutions of 1:5 thru 1:640, thus indicating that the correct serotypes were retained.

Polyclonal Antibody Production

New Zealand white rabbits, which were used to produce stock antisera for future use, gave variable results. Results of the serum neutralization (SN) tests, as described in Table 5, gave antisera titers as follows: SMSV-4, 1:160; SMSV-6, 1:80; SMSV-7, 1:640; SMSV-13, 1:5; MCV, 1:160; and RCV, 1:80.

Plaque Assay

The plaquing characteristics of the six serotypes are shown in Figure 1. Three plaque types were observed. Large, clear, round plaques seen for SMSV-13, developed after 24 to 48 hours of incubation and increased in diameter to 5 to 8 mm at 72 to 96 hours.

Figure 1. Cells were fixed with a 10% formalin solution and stained with crystal violet 3 days post-inoculation. A). Plaque morphology for the wild-type population of San Miguel Sea Lion Virus (SMSV), Type 4. Plaque size is 2 to 4 mm in diameter; B). (SMSV), Type 6, plaque size is 2 to 3 mm in diameter; C). (SMSV), Type 7, plaque size is 2 to 3 mm in diameter; D). (SMSV), Type 13, plaque size is 5 to 8 mm in diameter; E). Mink Calicivirus (MCV 20-3), plaque size is 1 to 2 mm in diameter; F). Reptile Calicivirus (RCV Cro-1), plaque size is 1 to 1.5 mm in diameter.

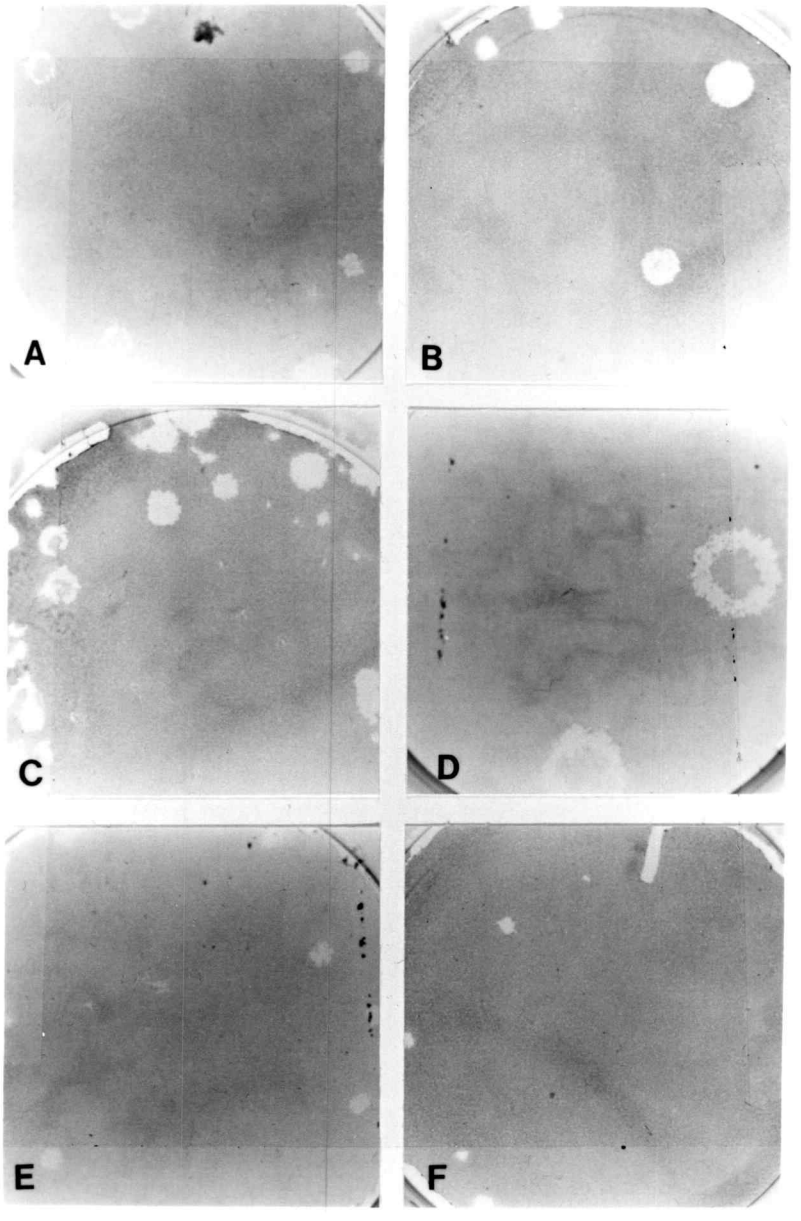


Figure 1. Plaqueing Characteristics

Intermediate, clear, irregular plaques were produced by SMSV serotypes 4,6 and 7, which ranged from 2 to 4 mm in diameter. RCV and MCV produced minute, opaque, irregular plaques, not visible until after 48 hours, and rarely larger than 1.5 mm in diameter, even after 5 to 7 days of incubation. Staining with neutral red (1:25 solution in sterile water) was necessary in order to visualize the minute plaques produced by MCV and RCV.

^{32}P-labeled Virus Purification

Purification of the ^{32}P-labeled virus yielded consistant, single, light-scattering bands that were shown by electron microscopy to contain virions with little or no cell debris.

Two-Dimensional PAGE of ^{32}P-Labeled Viral RNA

Electrophoresis in the first dimension results in mobilities dependent on the base composition. The uridine (U) rich residues have the most negative charge at pH 3.5, and migrate the fastest, followed by adenine (A) and then the cytosine (C) rich residues. Since ribonuclease T1 cleaves RNA on the 3' side of guanine (G) residues, each oligonucleotide should have a (G) nucleotide at its 3' end, except for the 3' end of the RNA genome. Loss of secondary and tertiary structure of the RNA occurs with increasing temperature during electrophoresis and with the addition of urea. Fingerprints of complete ribonuclease T1 digests of SMSV-4,6,7 and 13, MCV 20-3 and RCV Cro-1 are shown in Figures 2 thru 7, respectively. Migration in the first dimension was from left to right, beginning at the origin (0).

In the second dimension, each residue bears a charge of -1, and mobility is determined mainly by oligonucleotide size. By increasing the concentration of crosslinker in the second dimension, small oligonucleotides were prevented from migrating out of the gel, while simultaneously, all large ribonuclease T1 fragments remained well separated. Migration in the second dimension was from bottom to top. In each autoradiograph, the position of the two dye markers are indicated. The dark blue bromophenol blue (BB) dye is located in the upper center, and the lighter, blue-green, xylene cyanol (XC) dye is seen lower down. The fastest running material in the second dimension is the (Gp) residue. The fractionated oligonucleotides can be seen lying in a series of graticules, according to their (U) content, with progressive retardation in both dimensions by increasing contents of (A) and (C).

Autoradiography

Fingerprints, of each serotype are compared with respect to their oligonucleotide composition in Figures 8 thru 22. Oligonucleotides which are believed to be common to each serotype are shown as filled in spots. Oligonucleotides unique for each serotype are shown as empty spots. The area above the dashed line represents a mixture of oligonucleotides and was not compared, whereas all spots in the lower portion of the gel represent unique oligonucleotides. Lee and Wimmer (39) reported that prolonged electrophoresis of spots in the lower portion of gels does not yield furthur separation.

Figure 2. Autoradiograph of the two-dimensional polyacrylamide gel electrophoresis of Ribonuclease T1 resistant oligonucleotides of San Miguel Sea Lion Virus (SMSV), Type 4. The origin of electrophoresis (O) is located at the bottom left. The first dimension was carried out from left to right and the second dimension was carried out from bottom to top. The dye markers bromophenol blue (BB) and xylene cyanol (XC) are indicated. The Poly A tract (1) and Poly C tract (2) are also designated.

Figure 2. San Miguel Sea Lion Virus, Type 4.

Figure 3. Autoradiograph of the two-dimensional polyacrylamide gel electrophoresis of Ribonuclease T1 resistant oligonucleotides of San Miguel Sea Lion Virus (SMSV), Type 6. The origin of electrophoresis (O) is located at the bottom left. The first dimension was carried out from left to right and the second dimension was from bottom to top. The dye markers bromophenol blue (BB) and xylene cyanol (XC) are indicated. The Poly A (1) and Poly C (2) tracts are also designated.

Figure 3. San Miguel San Lion Virus, Type 6.

Figure 4. Autoradiograph of the two-dimensional polyacrylamide gel electrophoresis of Ribonuclease T1 resistant oligonucleotides of San Miguel Sea Lion Virus, Type 7. The origin of electrophoresis (0) is located at the bottom left. The first dimension was carried out from left to right and the second dimension was carried out from bottom to top. The dye markers bromophenol blue (BB) and xylene cyanol (XC) are indicated. The Poly A (1) and Poly C (2) tracts are also designated.

Figure 4. San Miguel Sea Lion Virus, Type 7.

Figure 5. Autoradiograph of the two-dimensional polyacrylamide gel electrophoresis of Ribonuclease T1 resistant oligonucleotides of San Miguel Sea Lion Virus, Type 13. The origin of electrophoresis (0) is located at the bottom left. The first dimension was carried out from left to right and the second dimension was carried out from bottom to top. The dye markers bromophenol blue (BB) and xylene cyanol (XC) are indicated. The Poly A (1) and Poly C (2) tracts are also designated.

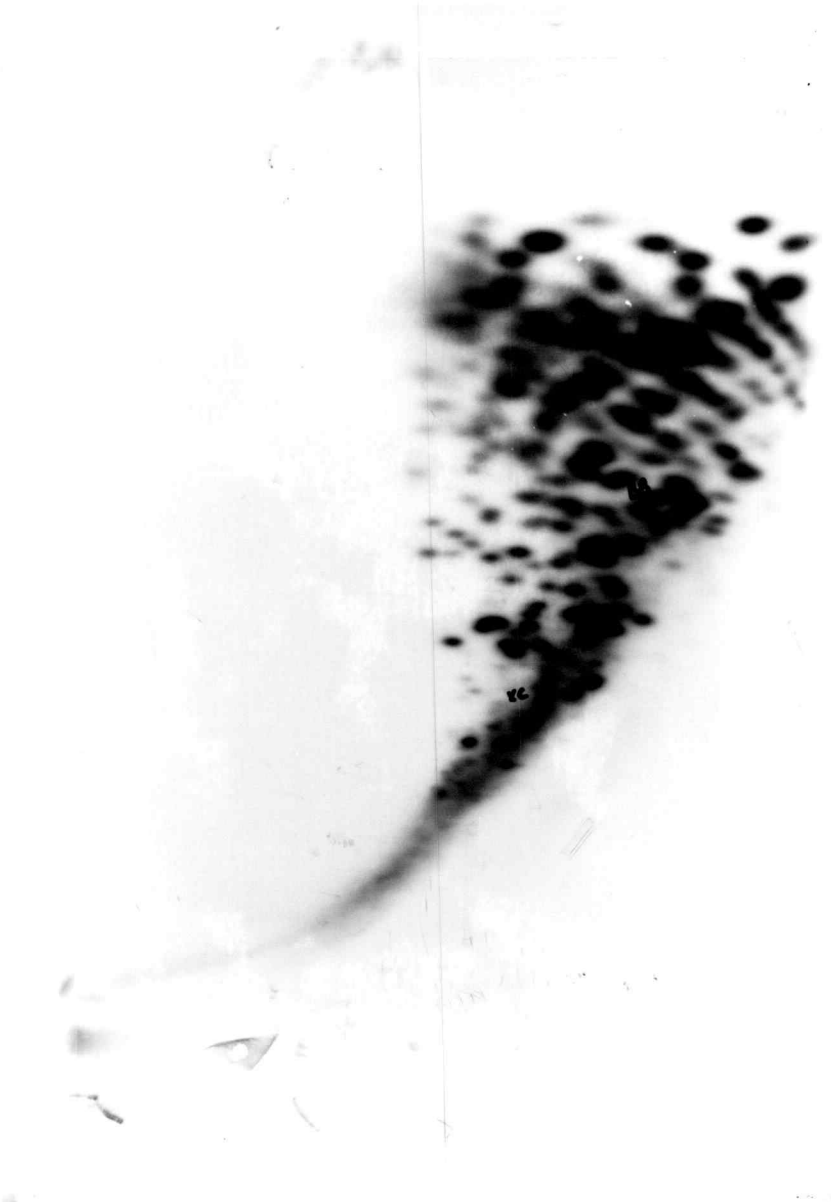


Figure 5. San Miguel Sea Lion Virus, Type 13.

Figure 6. Autoradiograph of the two-dimensional polyacrylamide gel electrophoresis of Ribonuclease T1 resistant oligonucleotides of Mink Calicivirus (MCV 20-3). The origin of electrophoresis (0) is located at the bottom left. The first dimension was carried out from left to right and the second dimension was from bottom to top. The dye markers bromophenol blue (BB) and xylene cyanol (XC) are indicated. The Poly A tract (1) and the Poly C tract (2) are also designated.

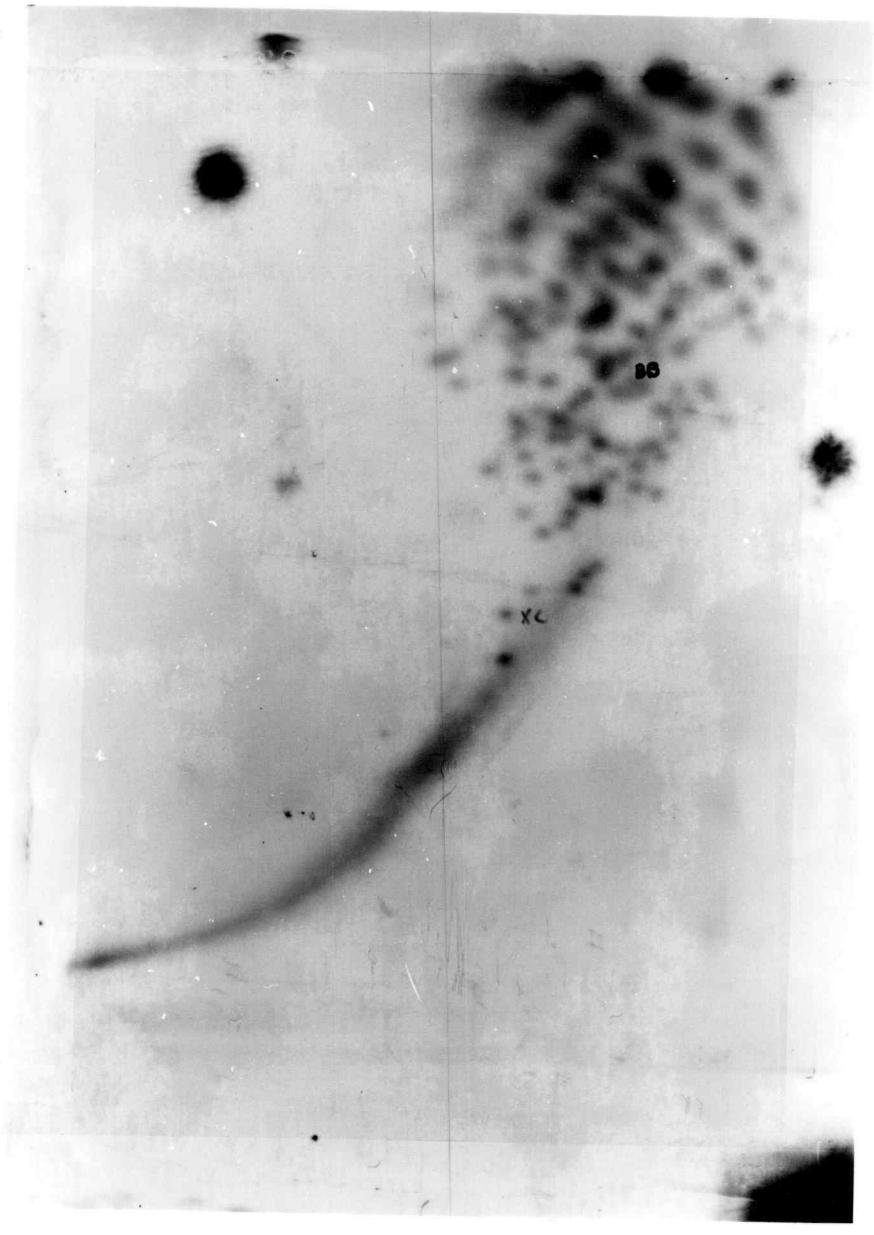


Figure 6. Mink Calicivirus (MCV 20-3).

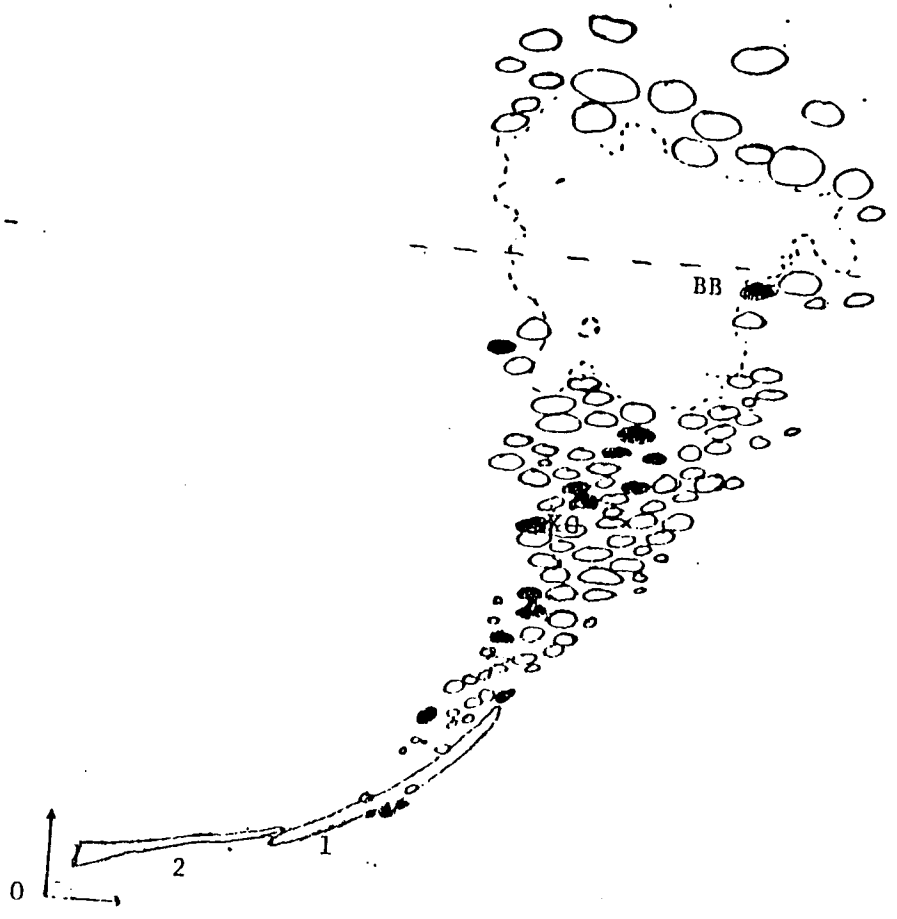
Figure 7. Autoradiograph of the two-dimensional polyacrylamide gel electrophoresis of Ribonuclease T1 resistant oligonucleotides of Reptile Calicivirus (RCV Cro-1). The origin of electrophoresis (0) is located at the bottom left. The first dimension was carried out from left to right and the second dimension was carried out from bottom to top. The dye markers bromophenol blue (BB) and xylene cyanol (XC) are indicated. The Poly A tract (1) and the Poly C tract (2) are also designated.

Figure 7. Reptile Calicivirus (RCV Cro-1).

Figure 8. Comparison of the two-dimensional polyacrylamide gel electrophoresis fingerprints of the Ribonuclease T1 resistant oligonucleotides of the wild-type population of San Miguel Sea Lion Virus (SMSV), Type 4, with the wild-type population of SMSV-6. Solid spots are oligonucleotides which are believed to be common to both serotypes. Open spots represent oligonucleotides unique to each serotype. The area above the dashed line was not compared. Both serotypes indicate the presence of a Poly A tract (1) and a Poly C tract (2). The diagrams for SMSV-4 and SMSV-6 were drawn from the autoradiographs in Figure 2 and Figure 3, respectively. The origin of electrophoresis is located at the bottom left (O). The first dimension of separation was carried out from left to right and the second dimension was from bottom to top. The position of the indicator dyes bromophenol blue (BB) and xylene cyanol (XC) are shown.

Figure 8.

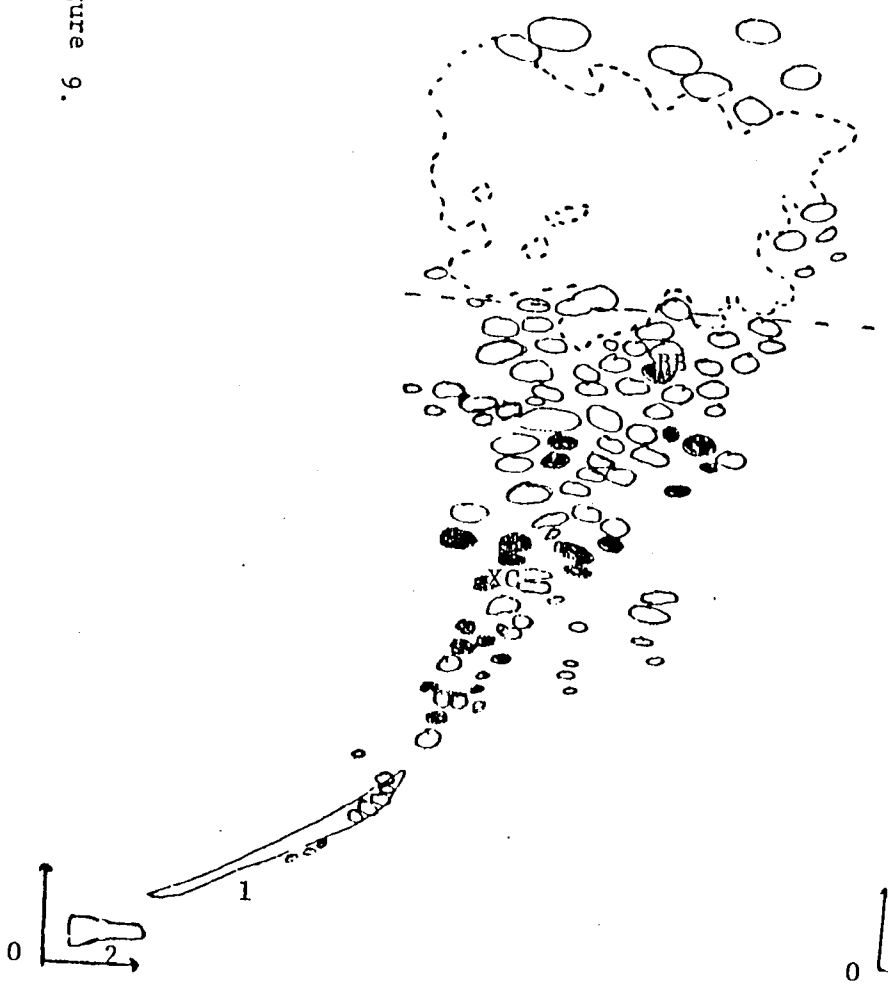
SMSV-4



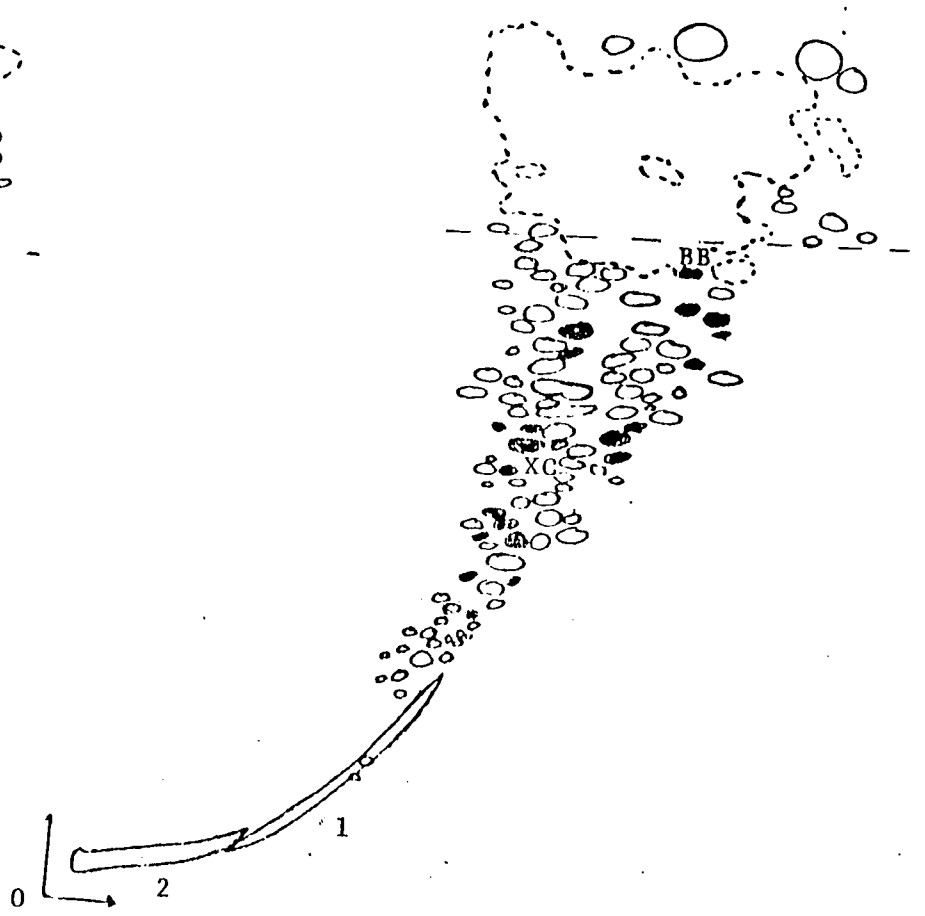
SMSV-6 (17 like spots)

Figure 9. Comparison of the two-dimensional polyacrylamide gel electrophoresis fingerprints of the Ribonuclease T1 resistant oligonucleotides of the wild-type population of SMSV-4, with the wild-type population of SMSV-7. Solid spots are oligonucleotides which are believed to be common to both serotypes. Open spots represent oligonucleotides unique to each serotype. The area above the dashed line was not compared. Both sertypes indicate the presence of a Poly A tract (1) and a Poly C tract (2). The diagrams for SMSV-4 and SMSV-7 were drawn from the autoradiographs in Figure 2 and Figure 4, respectively. The origin of electrophoresis is located at the bottom left (0). The first dimension of separation was carried out from left to right and the second dimension was from bottom to top. The position of the indicator dyes bromophenol blue (BB) and xylene cyanol (XC) are shown.

Figure 9.



SMSV -4

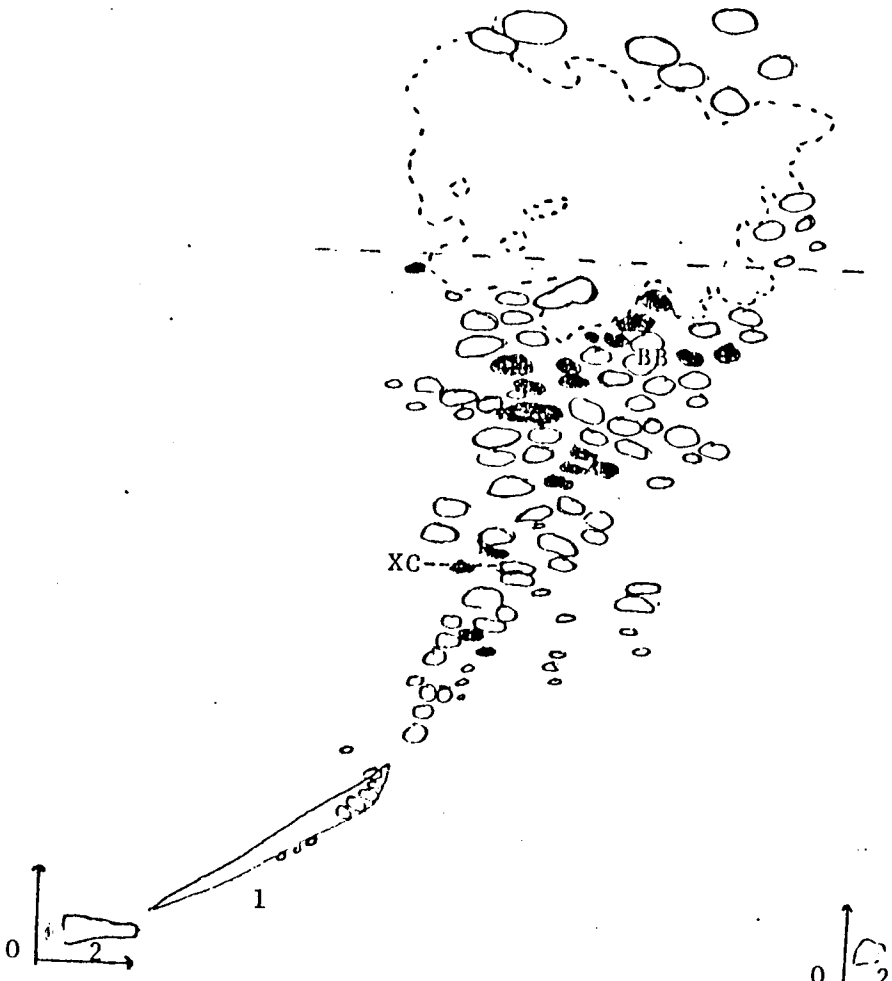


SMSV-7

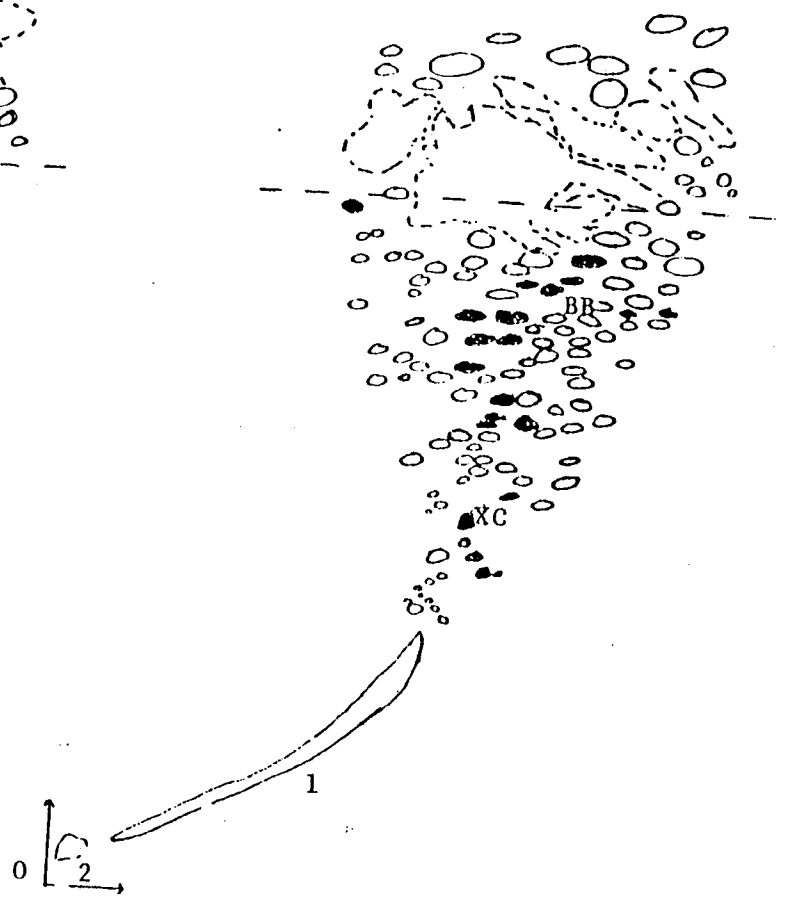
(20 like spots)

Figure 10. Comparison of the two-dimensional polyacrylamide gel electrophoresis fingerprints of the Ribonuclease T1 resistant oligonucleotides of the wild-type population of SMSV-4, with the wild-type population of SMSV-13. Solid spots are oligonucleotides which are believed to be common to both serotypes. Open spots represent oligonucleotides unique to each serotype. The area above the dashed line was not compared. Both serotypes indicate the presence of a Poly A tract (1) and a Poly C tract (2). The diagrams for SMSV-4 and SMSV-13 were drawn from the auto-radiographs in Figure 2 and Figure 5, respectively. The origin of electrophoresis is located at the bottom left (0). The first dimension of separation was carried out from left to right and the second dimension was from bottom to top. The position of the indicator dyes bromophenol blue (BB) and xylene cyanol (XC) are shown.

Figure 10.



SMSV-4

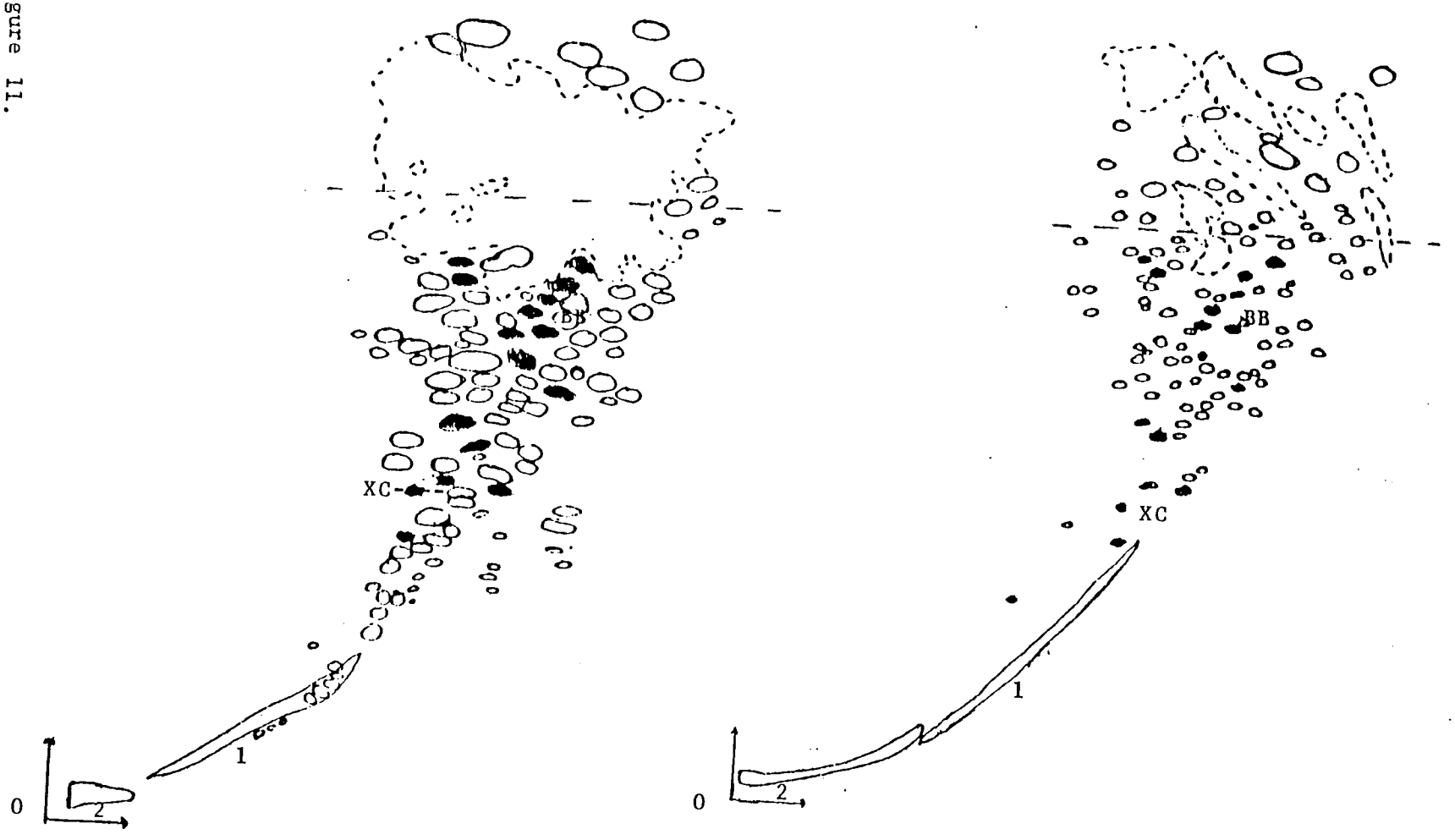


SMSV-13

(20 like spots)

Figure 11. Comparison of the two-dimensional polyacrylamide gel electrophoresis fingerprints of the Ribonuclease T1 resistant oligonucleotides of the wild-type population of SMSV-4, with the wild-type population of Mink Calicivirus (MCV 20-3). Solid spots are oligonucleotides which are believed to be common to both serotypes. Open spots represent oligonucleotides unique to each serotype. The area above the dashed line was not compared. Both viral types indicate the presence of a Poly A tract (1) and a Poly C tract (2). The diagrams for SMSV-4 and MCV 20-3 were drawn from the autoradiographs in Figure 2 and Figure 6, respectively. The origin of electrophoresis is located at the bottom left (0). The first dimension of separation was carried out from left to right and the second dimension was from bottom to top. The position of the indicator dyes bromophenol blue (BB) and xylene cyanol (XC) are shown.

Figure 11.



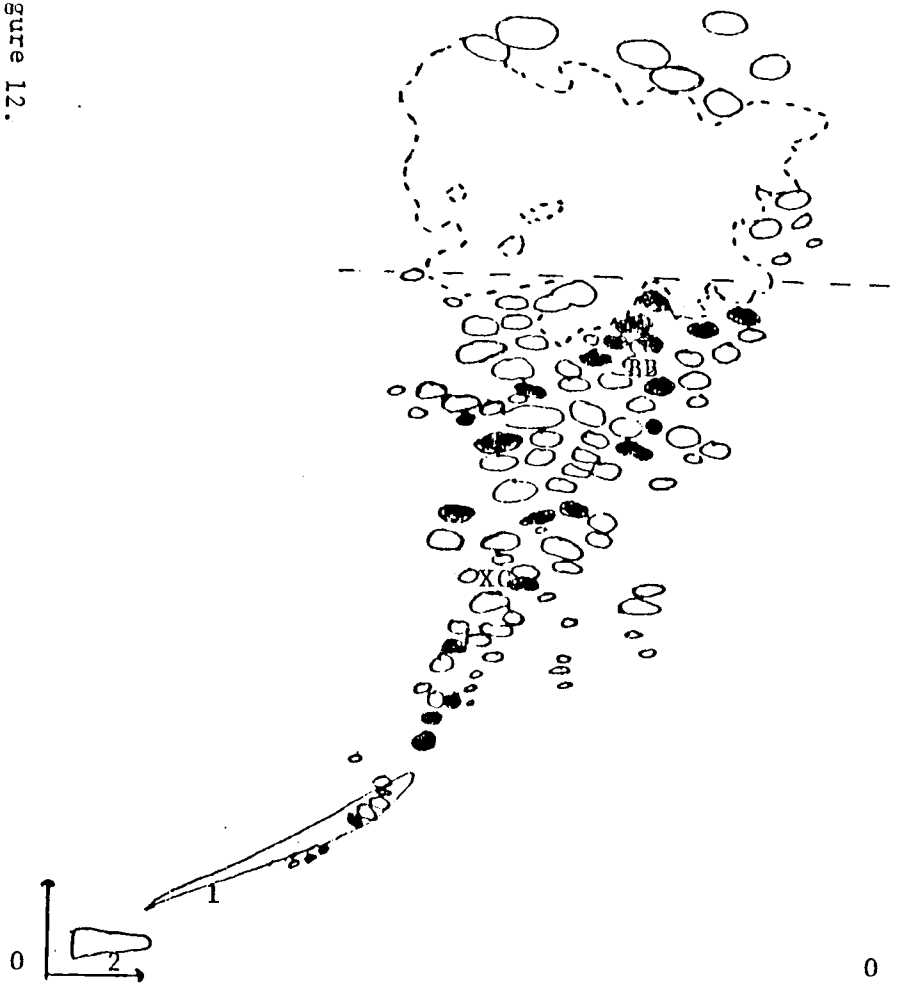
SMSV-4

(16 like spots)

Mink Calicivirus (MCV 20-3)

Figure 12. Comparison of the two-dimensional polyacrylamide gel electrophoresis fingerprints of the Ribonuclease T1 resistant oligonucleotides of the wild-type population of SMSV-4, with the wild-type population of Reptile Calicivirus (RCV Cro-1). Solid spots are oligonucleotides which are believed to be common to both serotypes. Open spots represent oligonucleotides unique to each serotype. The area above the dashed line was not compared. Both viral types indicate the presence of a Poly A tract (1) and a Poly C tract (2). The diagrams for SMSV-4 and RCV Cro-1 were drawn from the autoradiogrphs in Figure 2 and Figure 7, respectively. The origin of electrophoresis is located at the bottom left (O). The first dimension of separation was carried out from left to right and the second dimension was from bottom to top. The position of the indicator dyes bromophenol blue (BB) and xylene cyanol (XC) are shown.

Figure 12.

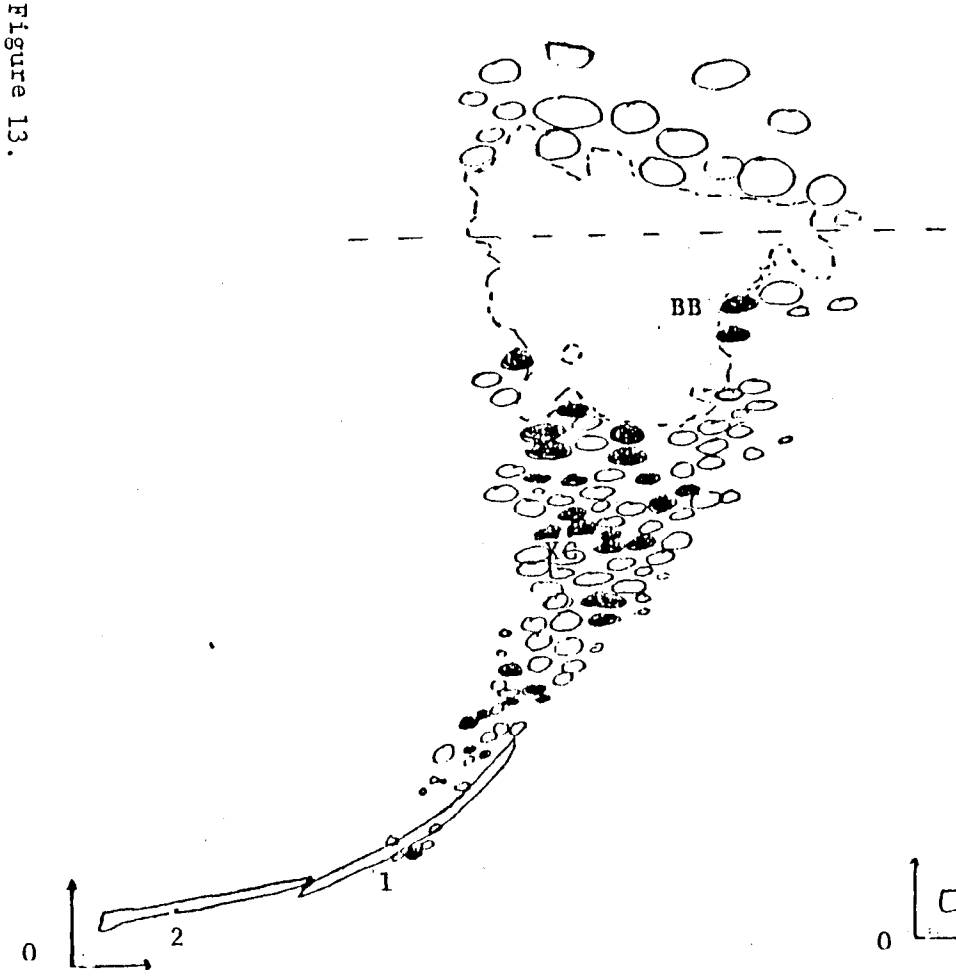


SMSV-4 (24 like spots)

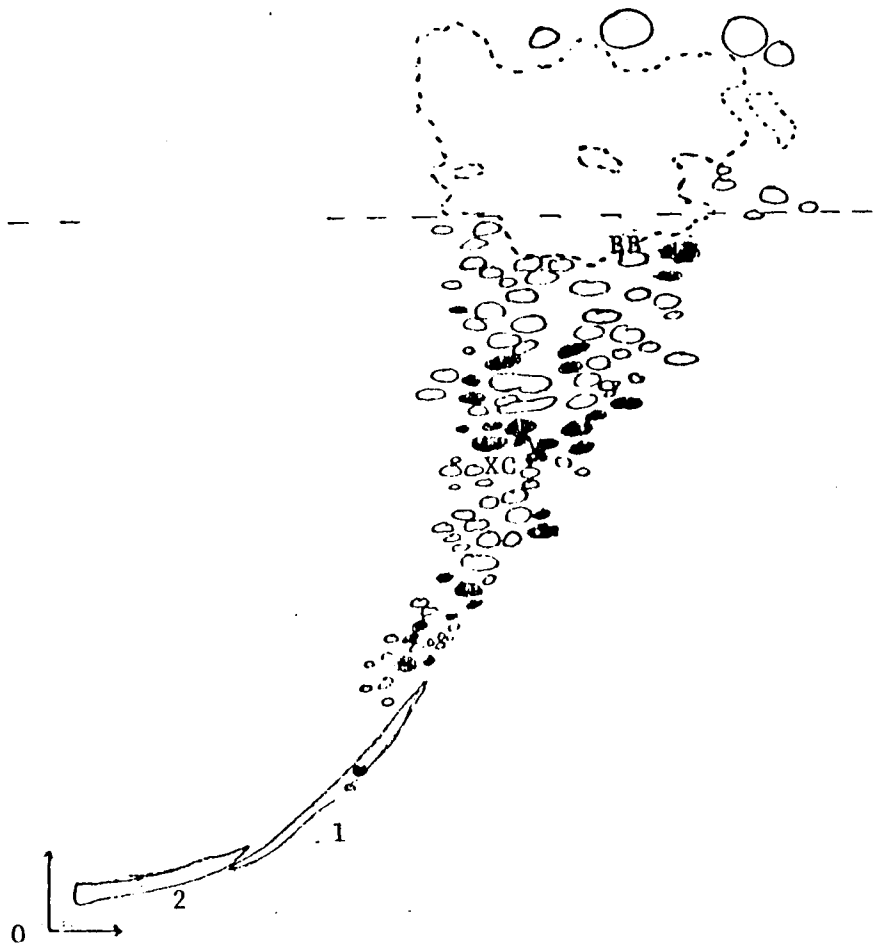
Reptile Calicivirus (RCV Cro-1)

Figure 13. Comparison of the two-dimensional polyacrylamide gel electrophoresis fingerprints of the Ribonuclease T1 resistant oligonucleotides of the wild-type population of SMSV-6, with the wild-type population of SMSV-7. Solid spots are oligonucleotides which are believed to be common to each serotype. Open spots represent oligonucleotides unique to each serotype. The area above the dashed line was not compared. Both serotypes indicate the presence of a Poly A tract (1) and a Poly C tract (2). The diagrams for SMSV-6 and SMSV-7 were drawn from the autoradiogrphs in Figure 3 and Figure 4, respectively. The origin of electophoresis is located at the bottom left (0). The first dimension of separation was carried out from left to right and the second dimension was from bottom to top. The position of the indicator dyes bromophenol blue (BB) and xylene cyanol (XC) are shown.

Figure 13.



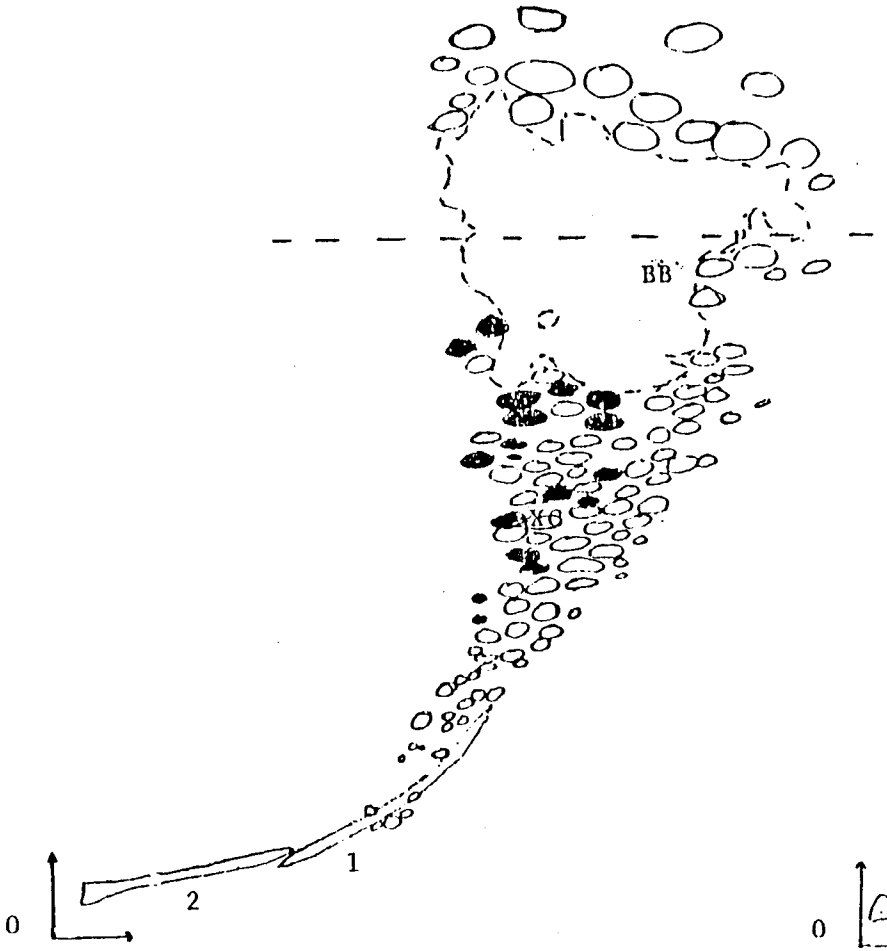
SMSV-6



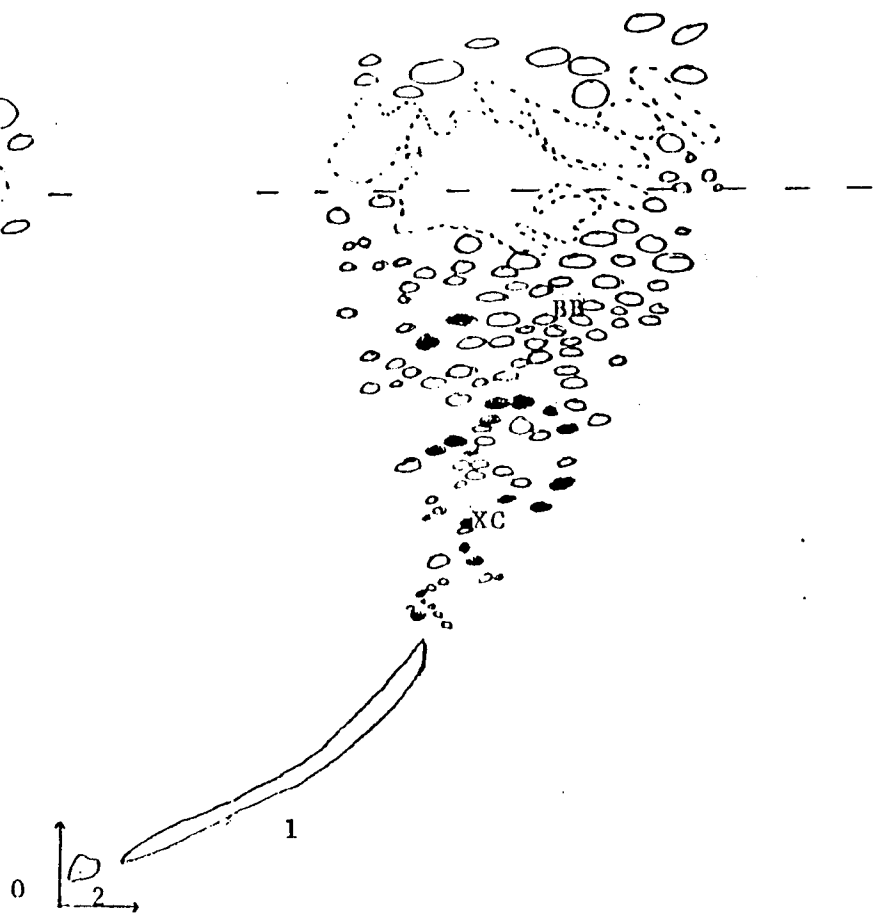
SMSV-7 (30 like spots)

Figure 14. Comparison of the two-dimensional polyacrylamide gel electrophoresis fingerprints of the Ribonuclease T1 resistant oligonucleotides of the wild-type population of SMSV-6, with the wild-type population of SMSV-13. Solid spots are oligonucleotides which are believed to be common to both serotypes. Open spots represent oligoonucleotides unique to each sero-type. The area above the dashed line was not compared. Both viral types indicate the presence of a Poly A tract (1) and a Poly C tract (2). The diagrams for SMSV-6 and SMSV-13 were drawn from the autoradiographs in Figure 3 and Figure 5, respectively. The origin of electrophoresis is located at the bottom left (0). The first dimension of separation was carried out from left to right and the second dimension was from bottom to top. The indicator dyes bromophenol blue (BB) and xylene cyanol (XC) are shown.

Figure 14.



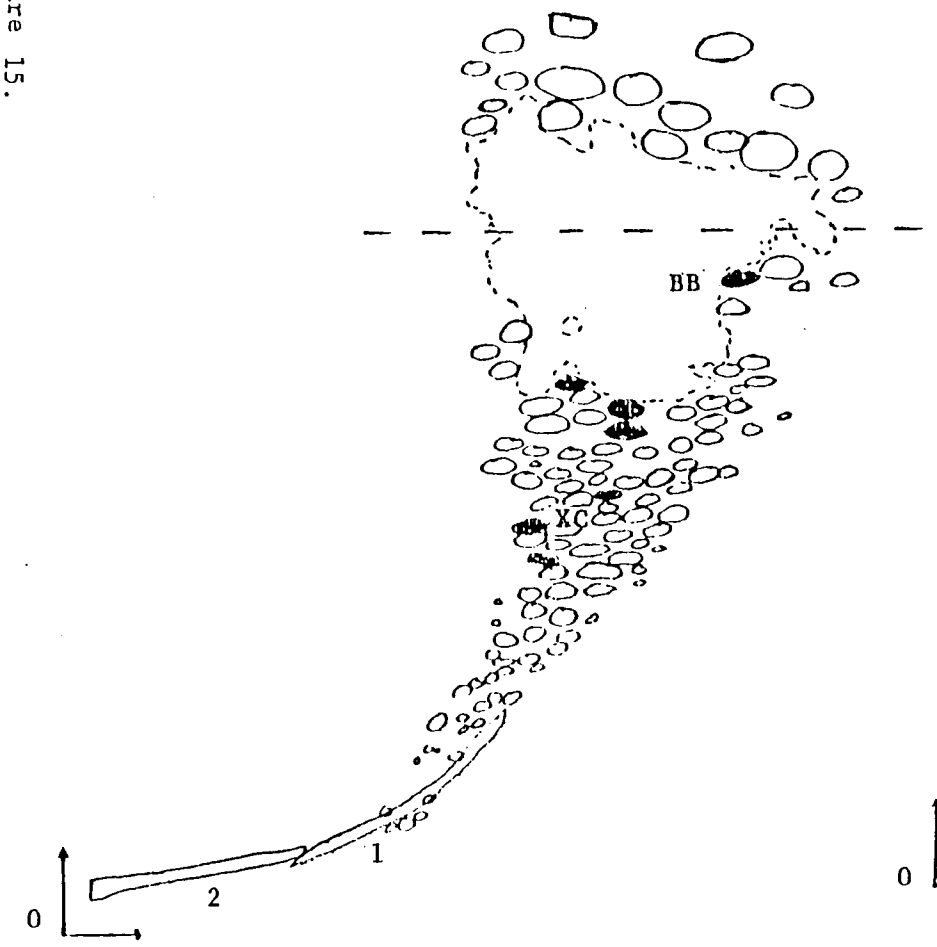
SMSV-6



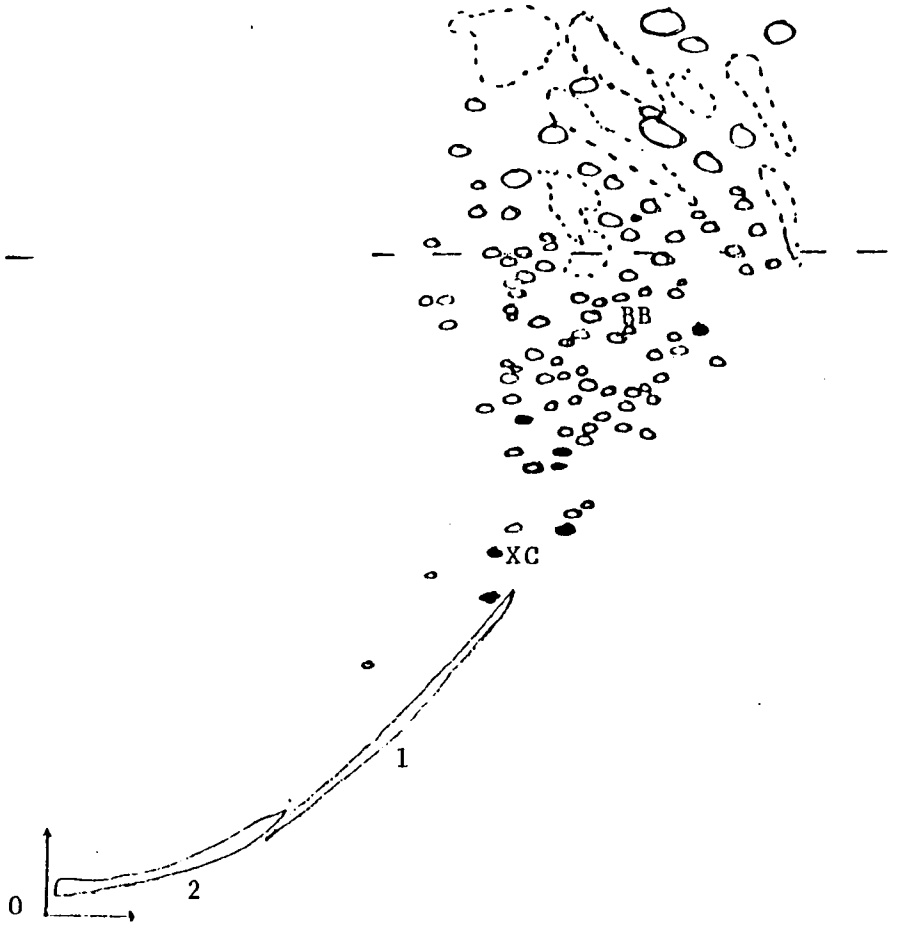
SMSV-13 (18 like spots)

Figure 15. Comparison of the two-dimensional polyacrylamide gel electrophoresis fingerprints of the Ribonuclease T1 resistant oligonucleotides of the wild-type population of SMSV-6, with the wild-type population of MCV 20-3. Solid spots are oligonucleotides which are believed to be common to both serotypes. Open spots represent oligonucleotides unique to each serotype. The area above the dashed line was not compared. Both serotypes indicate the presence of a Poly A tract (1) and a Poly C tract (2). The diagrams for SMSV-6 and MCV 20-3 were drawn from the autoradiographs in Figure 3 and Figure 6, respectively. The origin of electrophoresis is located at the bottom left (0). The first dimension of separation was carried out from left to right and the second dimension was from bottom to top. The position of the indicator dyes bromophenol blue (BB) and xylene cyanol (XC) are also shown.

Figure 15.



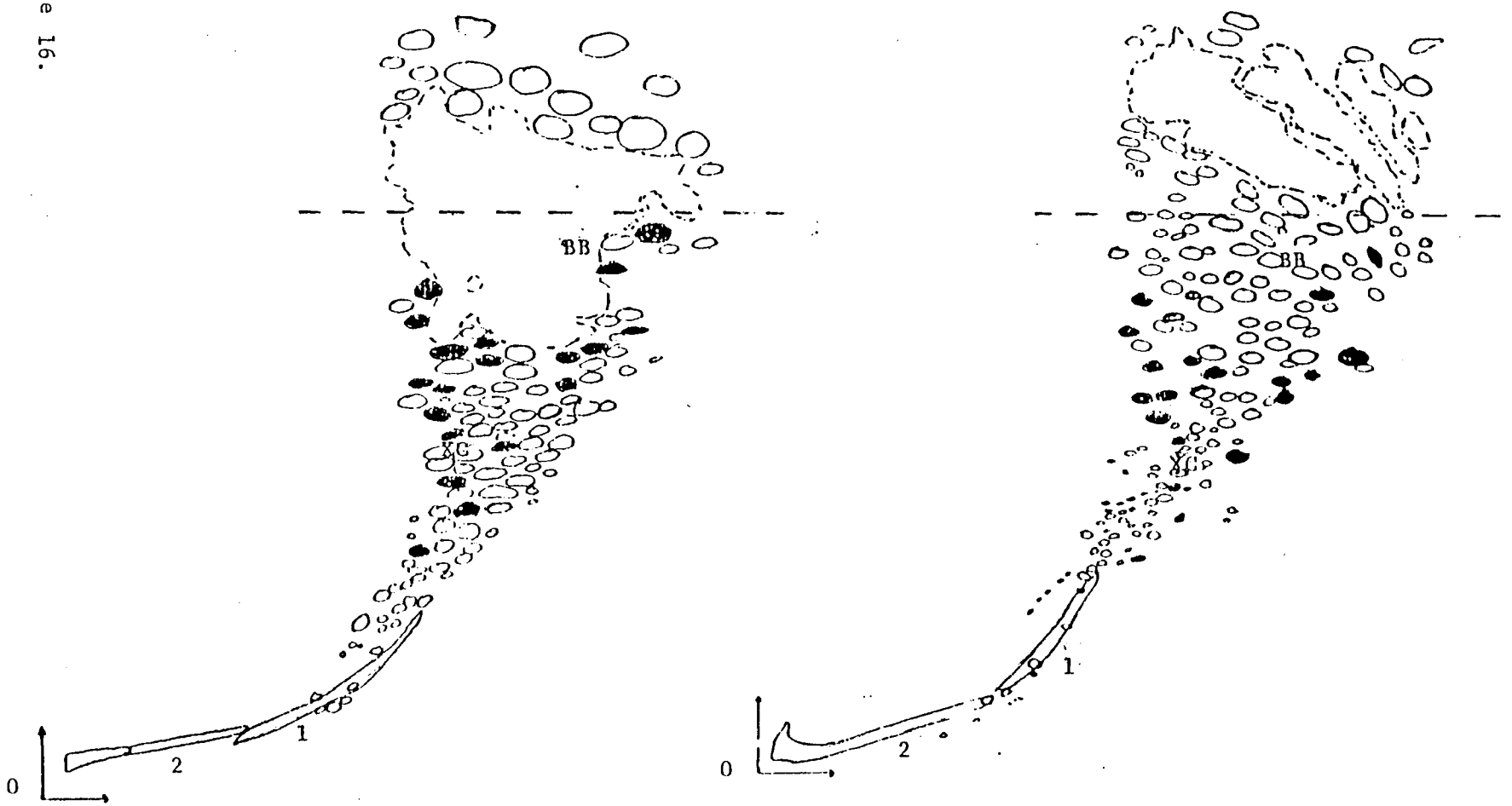
SMSV-6 (7 like spots)



Mink Calicivirus (MCV 20-3)

Figure 16. Comparison of the two-dimensional polycaryamide gel electrophoresis fingerprints of the Ribonuclease T1 resistant oligonucleotides of the wild-type population of SMSV-6, with the wild-type population of RCV Cro-1. Solid spots are oligonucleotides which are believed to be common to both serotypes. Open spots represent oligonucleotides unique to each serotype. The area above the dashed line was not compared. Both serotypes indicate the presence of a Poly A tract (1) and a Poly C tract (2). The diagrams for SMSV-6 and RCV Cro-1 were drawn from the autoradiographs in Figure 3 and Figure 7, respectively. The origin of electrophoresis is located at the bottom left (0). The first dimension of separation was carried out from left to right and the second dimension was from bottom to top. The position of the indicator dyes bromophenol blue (BB) and xylene cyanol (XC) are shown.

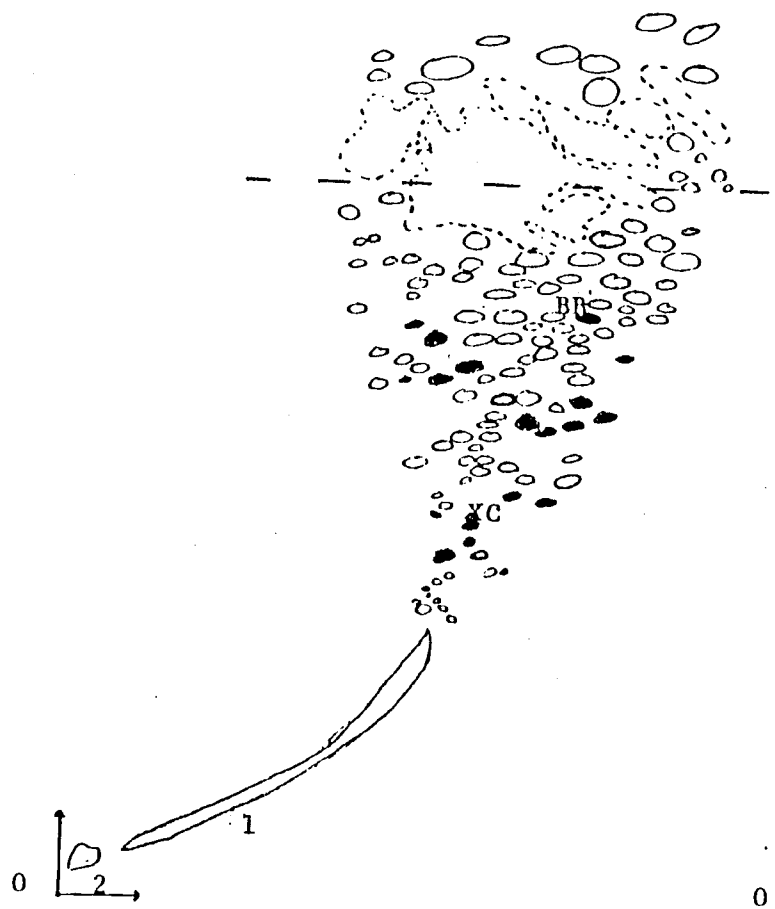
Figure 16.



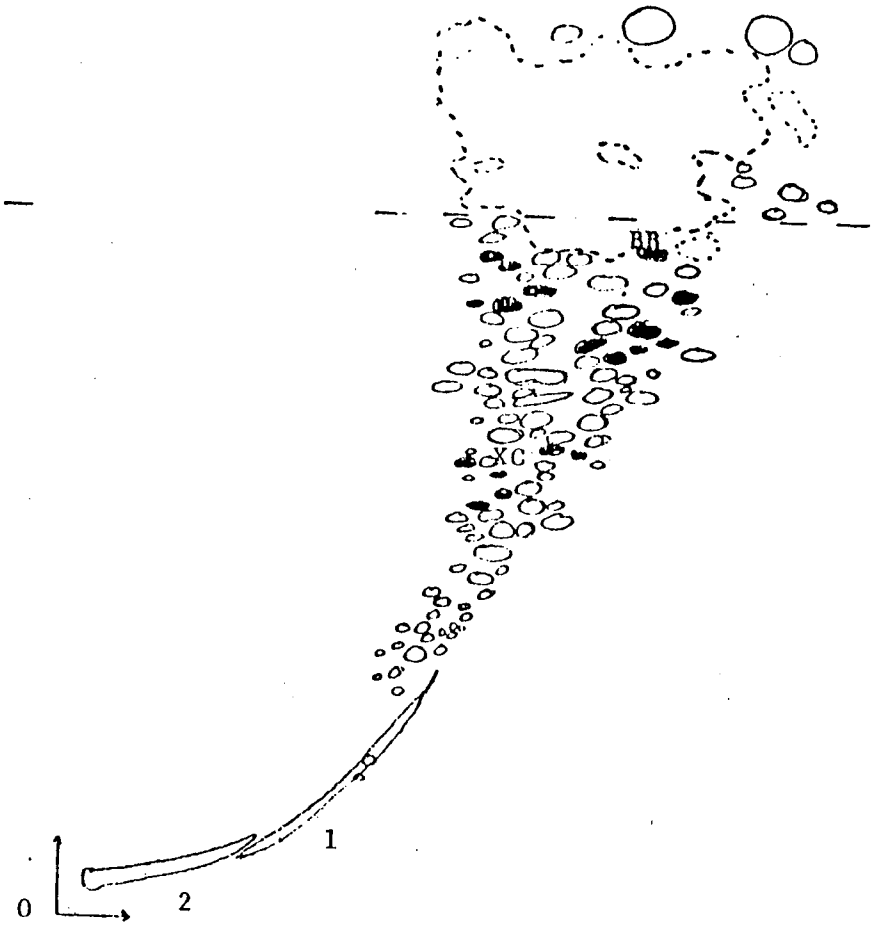
SMSV-6 (19 like spots) Reptile Calicivirus (RCV Cro-1)

Figure 17. Comparison of the two-dimensional polyacrylamide gel electrophoresis fingerprints of the Ribonuclease T1 resistant oligonucleotides of the wild-type population of SMSV-13, with the wild-type population of SMSV-7. Solid spots are oligonucleotides which are believed to be common to both serotypes. Open spots represent oligonucleotides unique to each serotype. The area above the dashed line was not compared. Both serotypes indicate the presence of a Poly A tract (1) and a Poly C tract (2). The diagrams for SMSV-13 and SMSV-7 were drawn from the autoradiographs in Figure 5 and Figure 4, respectively.The origin of electrophoresis is located at the bottom left (0). The first dimension of separation was carried out from left to right and the second dimension was from bottom to top. The position of the indicator dyes bromophenol blue (BB) and xylene cyanol (XC) are shown.

Figure 17.



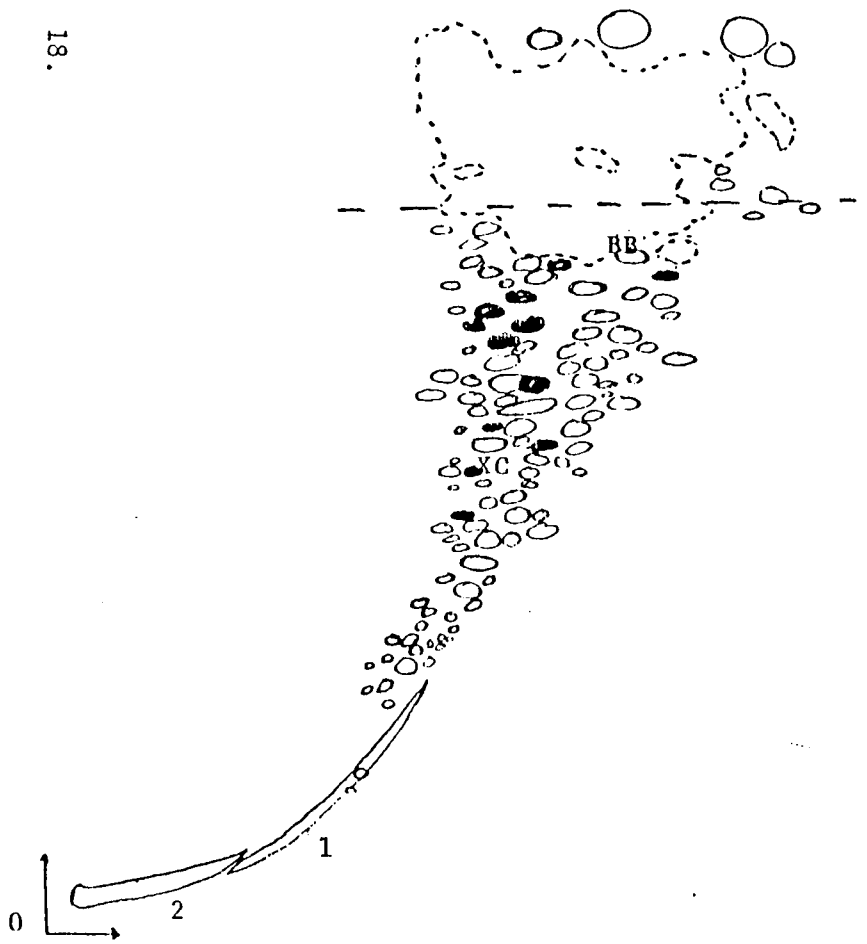
SMSV-13



SMSV-7 (18 like spots)

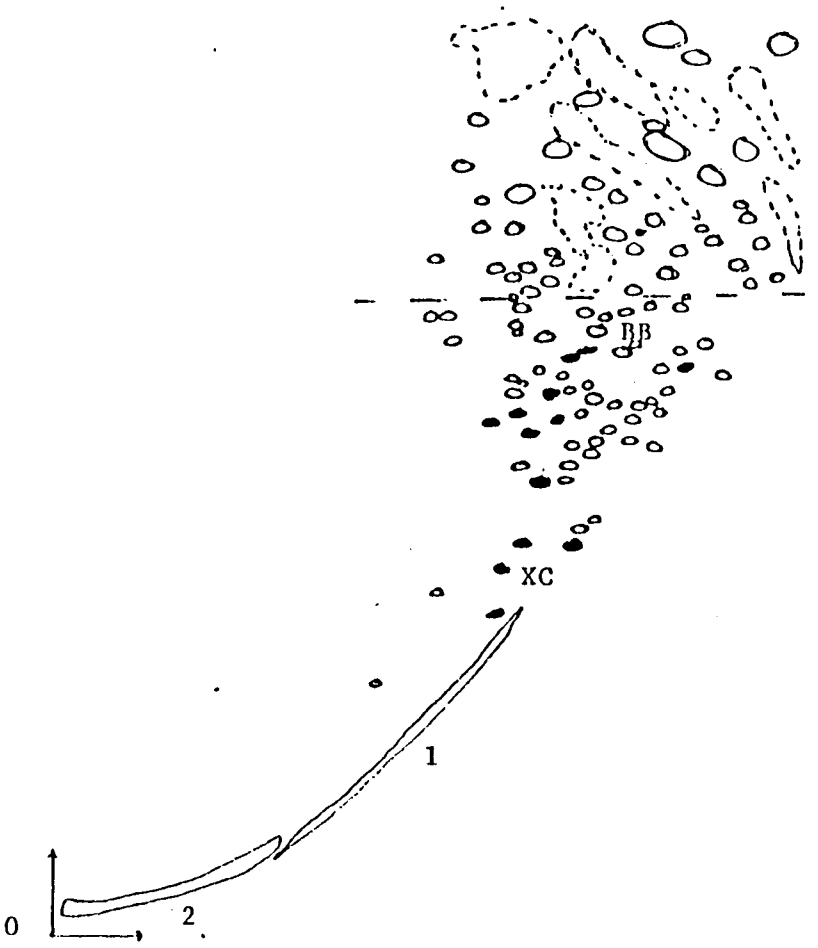
Figure 18. Comparison of the two-dimensional polyacrylamide gel electrophoresis fingerprints of the Ribonuclease T1 resistant oligonucleotides of the wild-type population of SMSV-7, with the wild-type populatiion of MCV 20-3. Solid spots are both serotypes. Open spots represent oligonucleotides unique to each serotype. The area above the dashed line was not compared. Both viral types indicate the presence of a Poly A tract (1) and a Poly C tract (2). The diagrams for SMSV-4 and MCV 20-3 were drawn from the autoradiographs in Figure 2 and Figure 6, respectively. The origin of electrophoresis is located at the bottom left (0). The first dimension of separation was carried out from left to right and the second dimension was from bottom to top. The position of the indicator dyes bromophenol blue (BB) and xylene cyanol (XC) are shown.

Figure 18.



SMSV-7

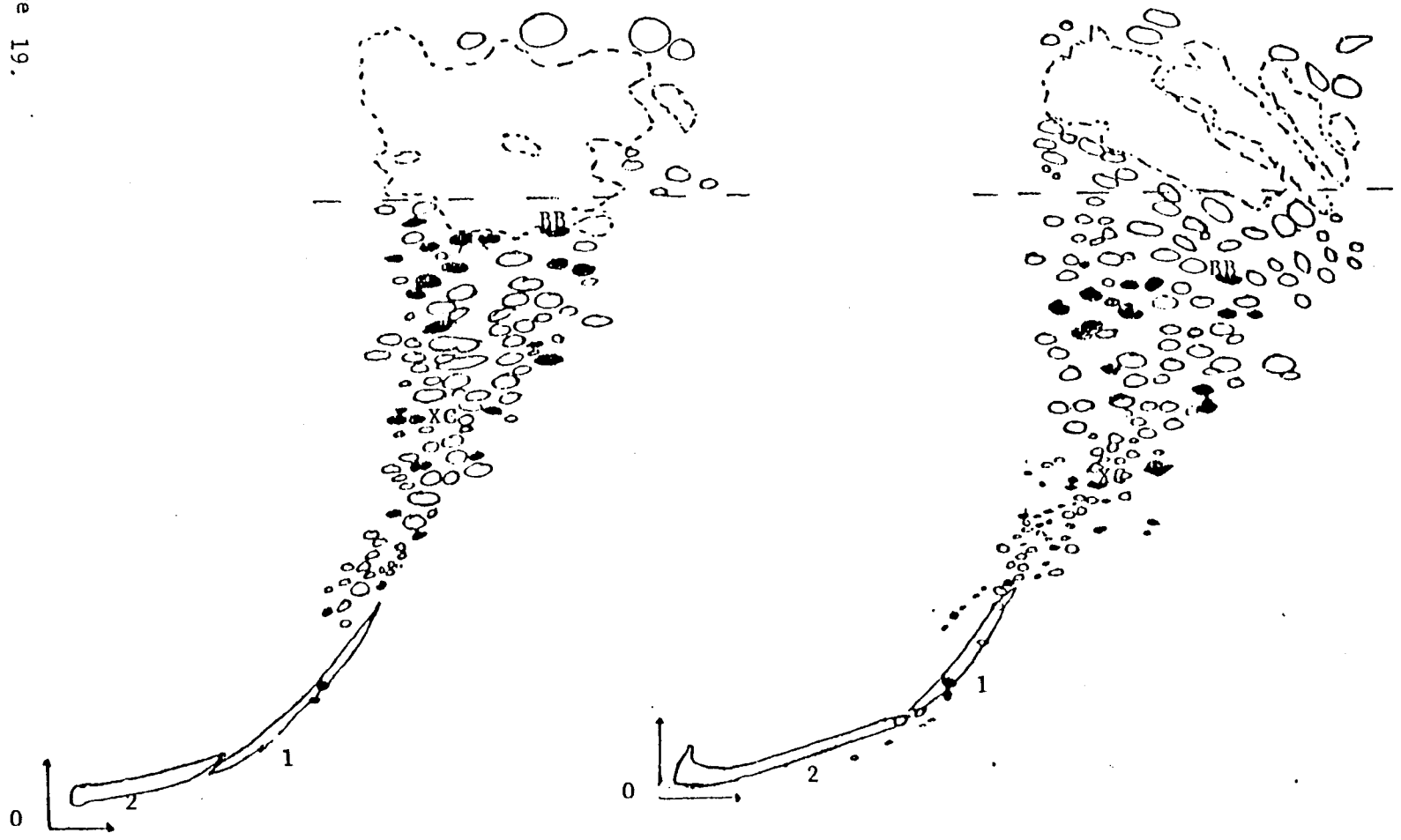
(12 like spots)



Mink Calicivirus (MCV 20-3)

Figure 19. Comparison of the two-dimensional polyacrylamide gel electrophoresis fingerprints of the Ribonuclease T1 resistant oligonucleotides of the wild-type population of SMSV-7, with the wild-type population of RCV Cro-1. Solid spots are oligonucleotides which are believed to be common to both serotypes. Open spots represent oligonucleotides unique to each serotype. The area above the dashed line was not compared. Both serotypes indicate the presence of a Poly A tract (1) and a Poly C tract (2). The diagrams for SMSV-7 and RCV Cro-1 were drawn from the autoradiographs in Figure 4 and Figure 7, respectively. The origin of electrophoresis is located at the bottom left (0). The first dimension of separation was carried out from left to right and the second dimension was from bottom to top. The position of the indicator dyes bromophenol blue (BB) and xylene cyanol (XC) are shown.

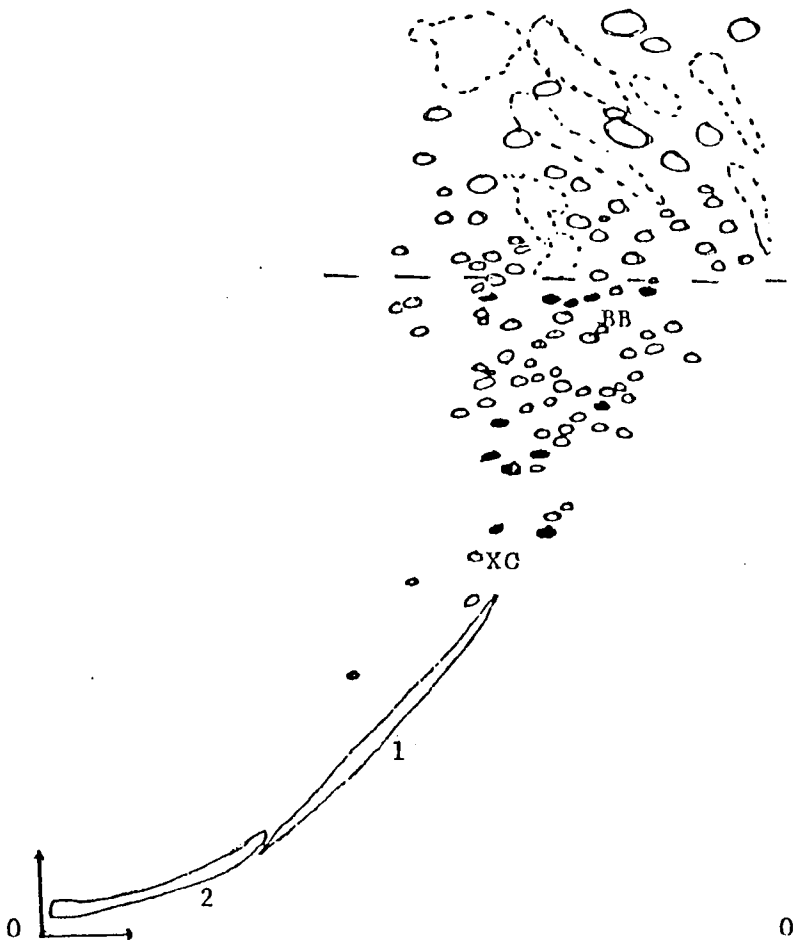
Figure 19.



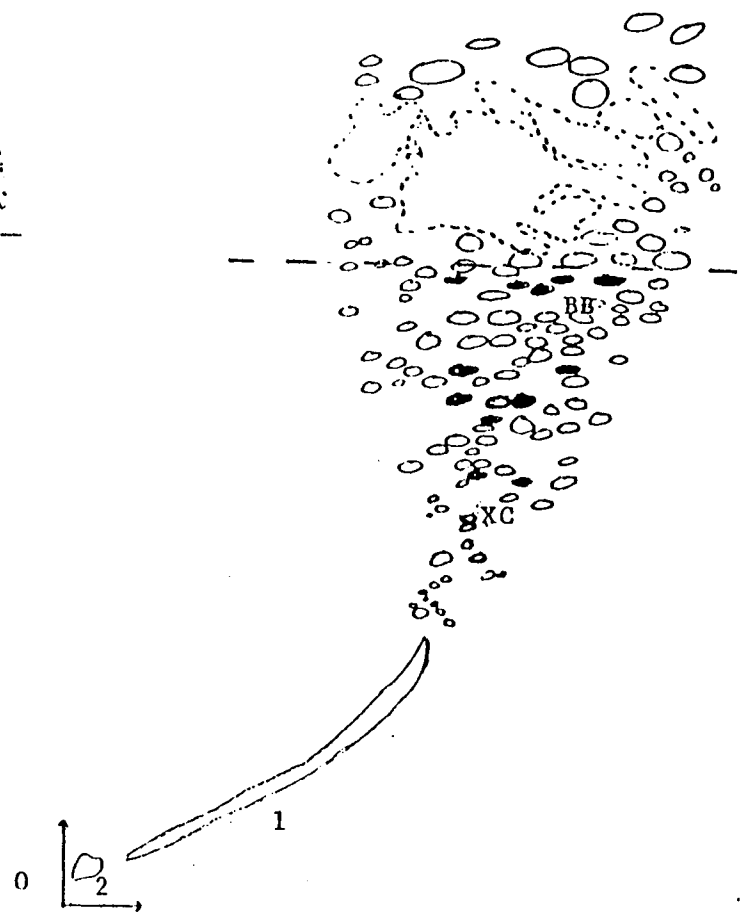
SMSV-7 (19 like spots) Reptile Calicivirus (RCV Cro-1)

Figure 20. Comparison of the two-dimensional polyacrylamide gel electrophoresis fingerprints of the Ribonuclease T1 resistant oligonucleotides of the wil-type population of Mink Calicivirus (MCV 20-3), with the wild-type population of SMSV-13. Solid spots are oligonucleotides which are believed to be common to both serotypes. Open spots represent oligonucleotides unique to each serotype. The area above the dashed line was not compared. Both serotypes indicate the presence of a Poly A tract (1) and a Poly C tract (2). The diagrams for MCV and SMSV-13 were drawn from the autoradiographs in Figure 6 and Figure 5, respectively. The origin of electrophoresis is located at the bottom left (0). The first dimension of separation was carried out from left to right anf the second dimension was from bottom to top. The position of the indicator dyes bromophenol blue (BB) and xylene cyanol (XC) are shown.

Figure 20.



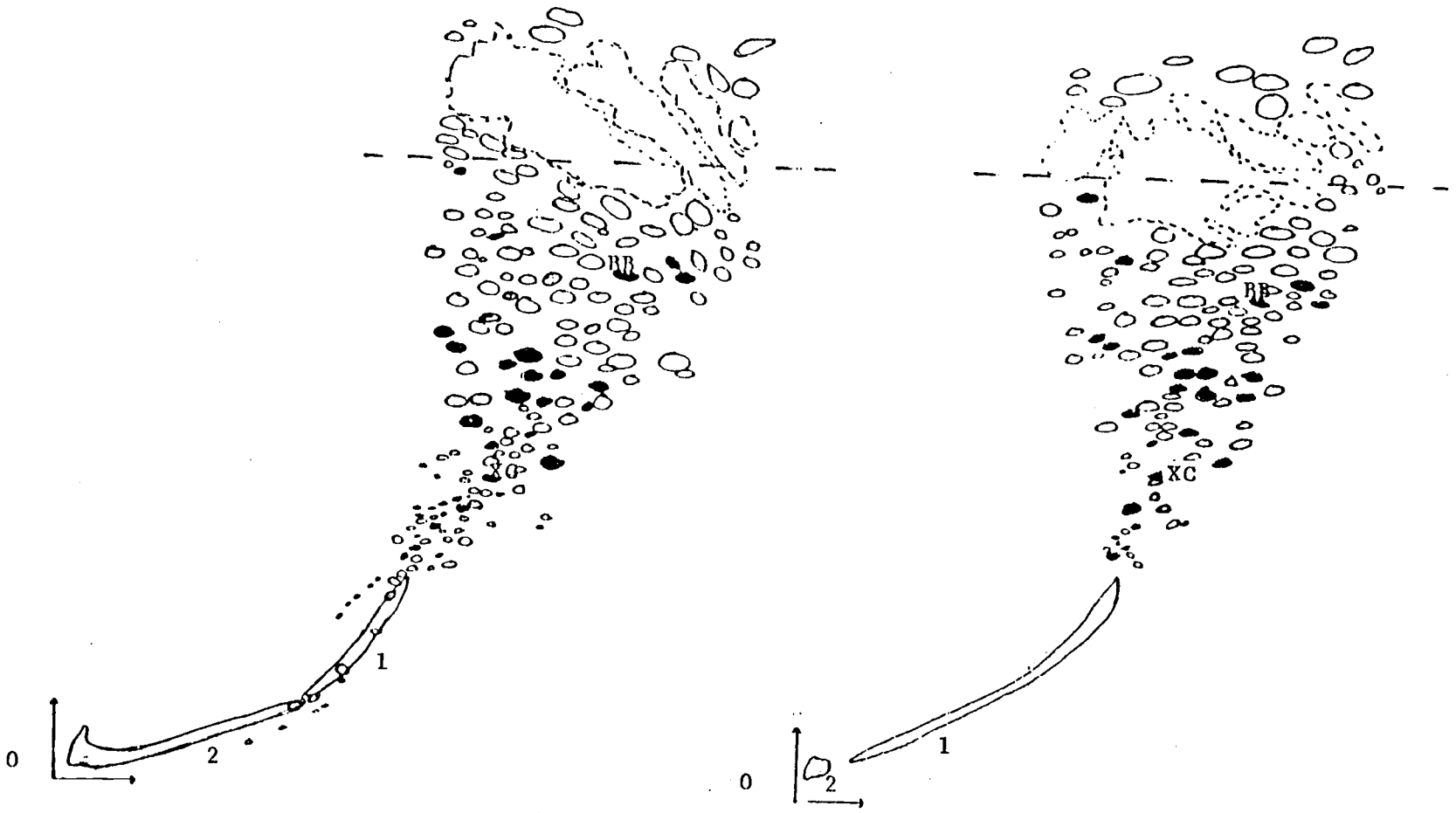
Mink Calicivirus (MCV 20-3)



SMSV-13 (12 like spots)

Figure 21. Comparison of the two-dimensional polyacrylamide gel electrophoresis fingerprints of the Ribonuclease T1 resistant oligonucleotides of the wild-type population of RCV Cro-1, with the wild-type population of SMSV-13. Solid spots are oligonucleotides which are believed to be common to both serotypes. Open spots represent oligonucleotides unique to each serotype. The area above the dashed line was not compared. Both serotypes indicate the presence of a Poly A tract (1) and a Poly C tract (2). The diagrams for RCV and SMSV-13 were drawn from the autoradiographs in Figure 7 and Figure 5, respectively. The orign of electrophoresis is at the bottom left (0). The first dimension of separation was carried out from left to right and the second dimension was from bottom to top. The position of the indicator dyes bromophenol blue (BB) and xylene cyanol (XC) are shown.

Figure 21.

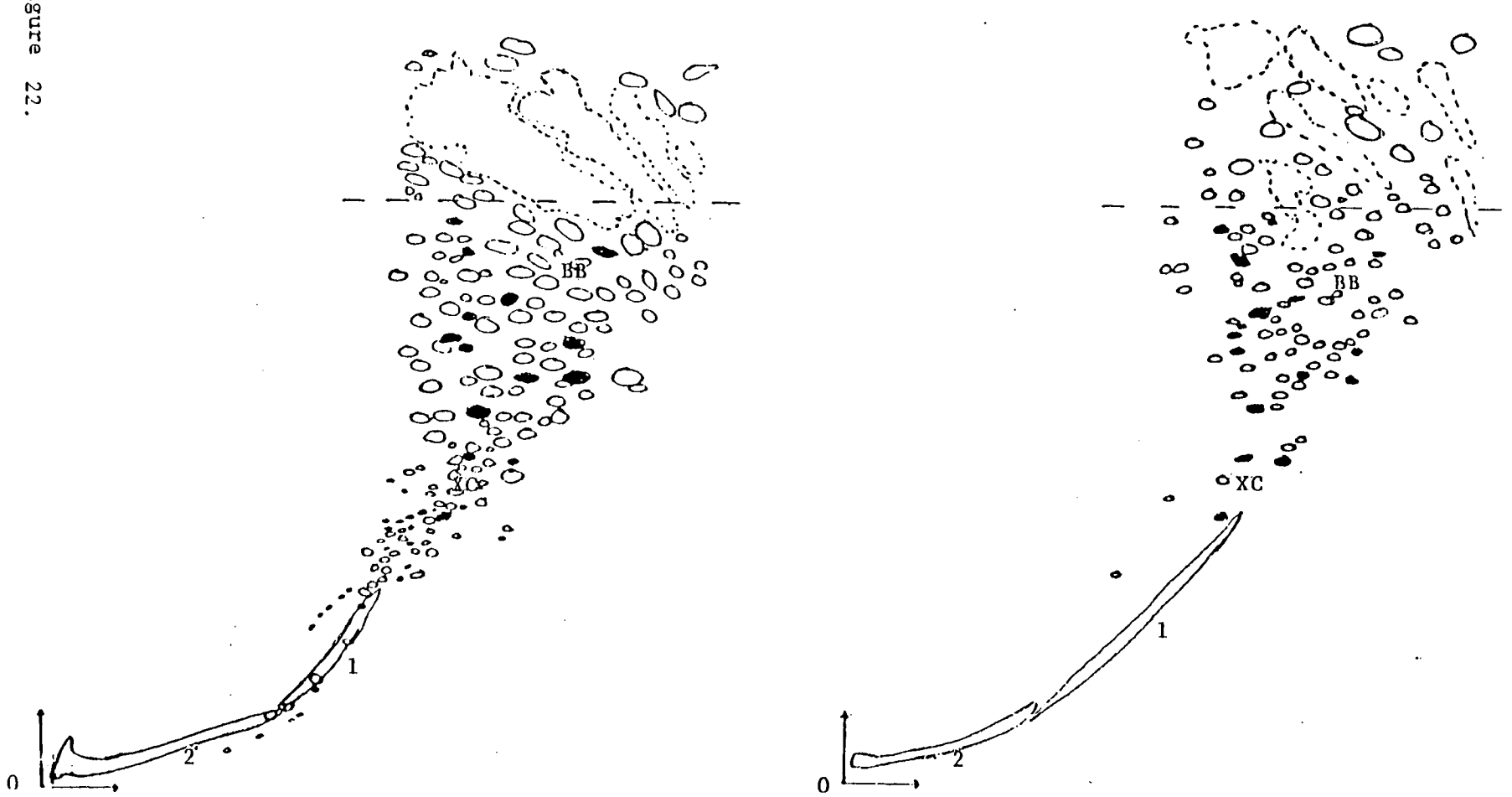


Reptile Calicivirus (RCV Cro-1)

SMSV-13 (24 like spots)

Figure 22. Comparison of the two-dimensional polyacrylamide gel electrophoresis fingerprints of the Ribonuclease T1 resistant oligonucleotides of the wild-type population of RCV Cro-1 with the wild-type population of MCV 20-3. Solid spots are oligonucleotides which are believed to be common to both serotypes. Open spots represent oligonucleotides unique to each serotype. The area above the dashed line was not compared. Both serotypes indicate the presence of a Poly A tract (1) and a Poly C tract (2). The diagrams of RCV and MCV were drawn from the autoradiographs in Figure 7 and Figure 6, respectively. The origin of electrophoresis is located at the bottom left (0). The first dimension of electrophoresis was carried out from left to right and the second dimension was from bottom to top. The indicator dyes bromophenol blue (BB) and xylene cyanol (XC) are shown.

Figure 22.



Reptile Calicivirus (RCV Cro-1) (14 like spots) Mink Calicivirus (MCV 20-3)

The presence of a Poly A tract (oligonucleotide #1) and a Poly C tract (oligonucleotide #2) was observed in each fingerprint. The Poly A oligonucleotides for each virus all appear to have a similar mobility, and therefore a similar size. However, mobility of the Poly A in polyacrylamide gels may not allow an accurate estimate of their length (34). Alkaline hydrolysis would need to be performed to determine the amount of adenylic acid present in these Poly A tracts.

The Poly C tract appears to vary in length from one serotype to the next. The Poly C oligonucleotides which migrate the furthest would theorhetically be of a smaller size while those rich in cytosine should not migrate as far due to their charge and size. The large radioactive spots remaining at the origin may indicate the presence of a Poly C oligonucleotide larger than 200 nucleotides. Lee and Wimmer (39) reported that no radioactive material remained in the first dimension of the gel when the chain length of the fragment was below 200 nucleotides.

Repeated two-dimensional gel electrophoresis fingerprints of SMSV-4 (3 replicates), SMSV-7 (2 replicates) and SMSV-13 (3 replicates) give identical results for each respective serotype, thus demonstrating the reproducibility of this technique when the conditions of temperature, polymerization time and degassing are followed.

DISCUSSION

Caliciviruses were originally classified as members of the family Picornaviridae (57,58). Like picorna-viruses, caliciviruses contain a VPg protein which is covalently bonded to the genomic RNA, and they lack a 5' methylated cap. However, caliciviruses differ from picornaviruses in that calicivirus VPg is required for RNA infectivity, whereas picornavirus VPg is not (18,59). The replication strategy of these viruses appears to resemble that of the alphaviruses (Togaviridae) in that two messenger RNA (m-RNA) species are synthesized in infected cells (29). All of the virus-specific RNA's have been shown to be polyadenylated, although the exact location of the Poly A tract on the genomic RNA has not been determined (29).

Wild-type populations of SMSV-4,6,7 and 13, MCV 20-3 and RCV Cro-1 were compared with each other (Figures 8 thru 22). The resulting fingerprints had a number of spots which, on the basis of their electrophoretic mobilities, appeared to be identical. Nevertheless, when the fingerprint for each serotype was examined, it was unique. The upper portion of the fingerprints were very similar to, but not identical to, each other. RNA's of high molecular weight can be expected to contain several small segments with similar base composition and chain length, therefore, RNase T1 digestion of the RNA will yield similar patterns in the upper portion of the gels. By superimposing the fingerprints, several oligonucleotides were shown to have similar mobilities and, therefore, possibly similar base composition.

Serum cross-neutralization studies demonstrated that each serotype was antigenically distinct (Table 5). Fingerprints of wild-type populations of SMSV-5 and SMSV-8 have been compared by Poet (52). Only 14 oligonucleotide spots, the Poly A tracts and the Poly C tracts were common to both serotypes. This lack of similarity between SMSV-5 and SMSV-8 supported serum neutralization studies by Smith, et al (73,82), which showed these two to be distinct serotypes.

SMSV-7 compared with SMSV-6 (Figure 13) shows the highest number (30 spots) of oligoucleotides with similar electrophoretic mobility. The Poly C tracts for SMSV-6 and SMSV-7, although not exactly the same length as determined by their mobilities, appear to be the nearest in size to each other, as compared to all of the other serotypes examined. At this time, it is not possible to determine how closely SMSV-6 and SMSV-7 are related. Cross-neutralization tests between these two isolates show no common reactivity at titers of 1:5. Both of these serotypes have been isolated from opaleye perch (82,80), and therefore may share some antigenic sites which are not detected in serum neutalization tests.

The fingerprints of MCV and RCV revealed the fewest similarities, when compared to other serotypes, possibly reflecting the phylogenetic divergence of the host species from which they were isolated.

Each fingerprint had what was believed to be an adenylic acid-rich tract (Poly A tract) (Figures 2 to 8). The presence of a Poly A sequence supports the theory that the viral RNA genome may act as an m-RNA inside of the cell. Ehresmann and Schaffer (29) identified Poly A tracts on the virus-specific RNA from SMSV-2 inoculated onto Vero cells. Both the 36S and 22S RNA's were polyadenylated, suggesting that

both RNA's serve a messenger function (29). The exact location of the Poly A sequence on the RNA genome has not been determined. Poly A tracts are found in the RNA of several members of the Picornaviridae family, as follows: poliovirus (3,39), Mengovirus (48), and FMD virus (36). The Poly A sequence in poliovirus and FMDV is 50 to 90 nucleotides long and is at the 3' end of the RNA molecule. The Poly A sequence of the Mengovirus is located at the 3' end, but there is some dispute as to the length, with values ranging from 15 to 70 nucleotides (48). Poly A tracts seen in Figures 2 to 8 appear to be similar in length based on their migration patterns, although the mobility of Poly A oligonucleotides in polyacrylamide gels may not provide accurate estimates of their length (39). Poet (52) reported that fingerprints of both large and minute plaque variants of SMSV-5 and SMSV-8 reveal Poly A tracts of similar length.

The slowest migrating large oligonucleotide in both the first and second dimensions is believed to be a Poly C oligonucleotide (Figures 2-8). Poly C tracts have been found in several FMDV RNA's (36), in encephalomyocarditis virus (EMCV) (53), and also in Mengovirus RNA (36). The Poly C tract ranges in length from about 100 nucleotides in EMCV RNA, to over 200 nucleotides in some serotypes of FMDV, and is composed of 80% to 90% (C) residues (15). The Poly C was located within the first 400 nucleotides of the 5' end for all FMDV isolates studied, and within 150 bases from the 5' end of the EMCV genome (13,36). The location of the Poly C sequence in calicivirus RNA is unknown.

Harris and Brown (35) performed oligonucleotide fingerprint analyses on a virulent and an avirulent strain of FMDV, serotype SAT1. The avirulent strain was derived from the virulent strain by serial

passage in BHK-21 cells and the two strains were shown to be physico-chemically and serologically identical. However, upon oligonucleotide fingerprint analysis, several differences were revealed, the most significant of these being the length of the Poly C tract. The Poly C oligonucleotides for the avirulent strain migrated furthur in both dimensions than that of the virulent strain, consistent with it being shorter in the avirulent virus RNA. There were approximately 170 nucleotides in the Poly C of the virulent strain compared with about 100 in the avirulent strain. This finding may establish a relationship between Poly C tract length and pathogenicity, but furthur studies are needed.

Studies by Poet (52) showed that the Poly C tract for large plaque variants of SMSV, Types 5 and 8 were longer than the Poly C tract for the minute plaque variants from the same serotypes, based on their electrophoretic mobilities in polyacrylamide gels.

Differences in the Poly C tracts, of the wild-type populations examined in this study are seen in Figures 2 to 8. The Poly C of SMSV-13 (Figure 5) mostly remained in the first dimension gel strip, indicating that it may contain over 200 cytosine residues (39). The Poly C of SMSV-4 (Figure 10) migrated only slightly further than the Poly C for SMSV-13 (Figure 10), and remained very close to the origin (0). SMSV-7 and SMSV-6 show Poly C tracts of similar size (Figure 13) as described earlier. The Poly C for MCV migrated further than all serotypes of SMSV examined (Figures 11,15,18,20), and the Poly C from RCV migrated further than the Poly C for MCV (Figure 22).

McClain, et al (46) performed swine infectivity studies using minute and large plaque variants from a single serotype of VESV. Large

plaque formers were found to be highly virulent, whereas minute plaque formers were either avirulent or had greatly reduced virulence. From the plaque populations observed for the serotypes used in the present study (Figure 1 A-F), and the migration of the Poly C (Figures 2 to 8), there appears to be a correlation between the plaque size and the length of the Poly C tract. SMSV-13 shows the largest plaque populations (5-8 mm), as well as the largest Poly C size. SMSV-4 has the second largest Poly C size, as well as the second largest plaque populations (2 to 4 mm). The Poly C sizes observed for SMSV-6 and SMSV-7 are approximately the same length, as determined by their mobilities, and their plaque populations show similar shape and size as well (2 to 3 mm). RCV and MCV consistently yielded populations of small plaques and in turn, their Poly C oligonucleotide migrated the furthest, indicating a small Poly C sequence in the RNA genome.

At present, the role of the Poly C sequence remains unknown. Harris and Brown (36) analyzed polypeptides of FMDV and found none of them to be rich in proline, suggesting that the Poly C sequence is untranslated. They furthur postulated that the Poly C sequence may function in RNA replication via a Poly G seqeunce in the negative replicative strand, which would initiate new strands of RNA of virus polarity. Soergel, et al (85) examined the amino acid composition of SMSV-2, and showed that it was not particularly rich in proline either, which suggests that the Poly C sequence in SMSV, like that of FMDV, may not be translated.

Since a relationship between plaque size and pathogenicity has been partially established (46,51,91), and the more virulent strain of an FMDV serotype has been shown to contain a larger Poly C sequence (35), a

relationship between calicivirus plaque size, Poly C migration, and virulence may exist and is consistent with the research reported herein. Furthurmore the size and migration patterns of the Poly C may serve as a valid indicator for determining future calicivirus pathotypes.

SUMMARY

Two-dimensional polyacryamide gel electrophoresis fingerprints of the Ribonuclease T1 resistant oligonucleotides of wild-type populations of SMSV-4,6,7 and 13, MCV 20-3 and RCV Cro-1 were compared. Similarities as well as differences between oligonucleotide patterns were observed, with SMSV-6 and SMSV-7 showing the most similarity. The presence of what is believed to be a Poly A tract and a Poly C tract was observed in each fingerprint. Whereas each of the Poly A tracts appear to be homogeneous in size, the length of the Poly C tracts differed for each serotype, as determined by the electrophoretic mobilities in the polyacrylamide gels. The results of this study show a direct relationship between plaque size and Poly C size for the six serotypes examined. Furthur studies, to determine the size and composition of the Poly C will be necessary before the Poly C length, as seen in the polyacrylamide gels, can be used for reliably differentiating calicivirus pathotypes.

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