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Title: Prevalence and Distribution of Calicivirus Neutralizing Antibody in Selected U.S. Cattle

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Serum samples from 1,046 Kansas cattle were examined for the presence of neutralizing antibody to caliciviruses known to be circulating in the marine environment. All sera were from farm-origin cattle, with the number tested being proportionate to the total number of cattle in each county. These sera were selected in a random manner from blood samples routinely submitted to the Kansas State/Federal Brucellosis Laboratory in 1988. The majority of animals represented by the sample were adult females of mixed breed. The sera were tested for the presence of antibody to the San Miguel sea lion virus (SMSV) types; 1, 5, 7, 8, 14, and the Tillamook calicivirus serotype (BCV Bos-1). Antibody was detected by the microneutralization procedure with Vero cells as the indicator system. Using a 1:10 serum dilution, neutralizing antibodies were found in the sample to all serotypes except SMSV-1 with prevalence rates ranging from .3% to 32%. All neutralization screen tests were read to a 100% end point (EP) using a 100-421 tissue culture infective dose 50% end point ($TCID_{50}$) of challenge virus. Due to the low antibody levels, prevalence values were found to be very dependent upon the virus challenge dose and the

antibody EP used in the test. Antibody titers to four serotypes ranged from 1:10 to 1:1280 with most falling in the 1:10 to 1:20 range using a 50% antibody EP and a 100-421 $TCID_{50}$ of challenge virus. Several sera were found to possess neutralizing antibody to multiple serotypes. Antibody to SMSV-5 was shown to be well distributed throughout Kansas indicating that widespread exposure to this marine calicivirus had occurred.

Prevalence and Distribution of Calicivirus Neutralizing Antibody in Selected U.S. Cattle

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OBJECTIVE

The objective of this study is to determine the prevalence and distribution of calicivirus exposures in Kansas cattle. Six calicivirus serotypes isolated from the marine environment and a seventh type found to be present in the marine environment and assumed to be of marine origin were selected for study. Serum samples of indigenous Kansas cattle were selected on a random basis from a collection of blood samples submitted for Brucellosis screening in Kansas. A proportional number of samples were selected from each county on the basis of current cattle census data. The serum neutralization assay is used to detect calicivirus antibody for serological evidence of exposure. It is thought that prevalence of exposure found in Kansas cattle will provide conservative estimates of exposure for other U.S. cattle as the state is geographically removed from direct ocean sources of virus. Characterization of the distribution of exposure in Kansas may provide insights into the distribution of these agents in other states with similar cattle movements and husbandry practices. Prevalence and distribution data generated by this study is combined with the existing knowledge of calicivirus movements in marine and terrestrial environments to produce a better understanding of calicivirus epidemiology and potential disease impacts on domestic livestock.

INTRODUCTION

Caliciviruses have unique characteristics that set them apart from other virus families. These viruses were initially classified as a separate genera within the picornavirus family; however, significant differences have become apparent after more complete characterization. Reclassification has placed these agents in a separate family named caliciviridae consisting of one genus designated calicivirus. Characteristics that distinguish these viruses from picornaviruses and other features of this virus family have been summarized (20, 60, 69, 122).

Caliciviruses are small, round, non-enveloped viruses with single stranded, non-segmented, positive sense RNA and have icosahedral symmetry. The surface of the virion is cupped giving it a spiked appearance in contrast to the smooth spherical morphology of picornaviruses. These viruses have a diameter of between 35 and 40 nanometers, molecular weight (MW) of 14 x 10^6 daltons, buoyant density of 1.36-1.39 gm/ml in cesium chloride and a sediment coefficient of 170-183S in sucrose gradients. The caliciviruses are, therefore, significantly larger than picornaviruses (60, 69).

Caliciviruses are composed of 80% protein and 20% RNA and possess a protein coat. The capsid is composed of one structural polypeptide in contrast to several polypeptides making up the protein coat of picornaviruses. The single structural protein of caliciviruses is translated from subgenomic RNA (1 x 10^6 MW) acting as its own messenger. In comparison, picornavirus proteins are produced from the cleavage of a

polyprotein coded by a full-length genomic messenger RNA. The non-structural proteins of caliciviruses differ from those of picornaviruses and are coded for by full-genomic-sized RNA (2.1 x 10^6 MW). Both virus families are similar in that a protein necessary for infectivity (VPg) is covalently bound to viral RNA. The cytoplasm is the site of calicivirus protein accumulation and viral maturation. Viral crystalline arrays, linear arrays and noncrystalline arrays frequently form in a variety of cell lines (60, 69).

The size of viral RNA is approximately the same for both virus families. Three calicivirus RNA species have been characterized, with sedimentation coefficients of 32S, 22S and 18S. The 32S species represents complete genomic RNA and the 22S species is responsible for polyprotein translation. The base composition of calicivirus RNA is A:27-29%, C:22-24%, G:22% and U:26-27% (20, 69).

Caliciviruses are relatively resistant as viral replication is unaffected by cell inhibitors such as; actinomycin D, halogenated uridine, deoxyribosides, guanidine and hydroxybenzyl benzimidazole. These viruses are inactivated rapidly at 50°C and not heat-stabilized by divalent cations such as Mg++ and Ca++. In addition, caliciviruses are not resistant to a pH of 3 or less, and can be inactivated by ultraviolet radiation; however, they are not sensitive to lipid solvents (60, 69, 122). Indefinite survival in buffered solutions at 4°C in the presence of L-cysteine has been noted (74) and these viruses, when compared to the picornaviruses, exhibit different survival characteristics in chemical containing aerosols (41).

Although the above features are shared by all members of the calicivirus family, there are antigenically distinct types as determined

by animal inoculation cross-immunity and virus neutralization tests. Each antigenic type has been classified as a new serotype and Cubitt (32) has divided these serotypes into two general groups.

The first group has been designated the enteric candidate caliciviruses (Table 1). These agents have been recovered from hosts with gastroenteritis and do not possess the in vitro growth patterns of other caliciviruses. Members of the enteric candidate calicivirus group include the Norwalk agent, human calicivirus strains (HCV), Newbury agent of cattle, porcine enteric calicivirus (PEC), canine calicivirus (CaCV) and the chicken calicivirus (CCV). The CaCV however appears to be related antigenically to the CCV (71). Although not readily associated with enteric disease, the Amyelosis chronic stunt virus (ACSV), recovered from hemocytes of moribund navel orange worm larvae (52), is considered a member of this candidate group. Preliminary work with the virus of non-A, non-B hepatitis indicates that it also should be classified as an enteric candidate calicivirus (22, 23).

The second group has been defined by Cubitt as the characterized caliciviruses. This grouping includes the serotypes of vesicular exanthema of swine virus (VESV), the San Miguel sea lion viruses (SMSV's) and the strains of feline calicivirus (FCV).

For the purpose of this study, the characterized group is further divided on the basis of demonstrated marine presence. Included in the marine group are the VESV's, SMSV's, and the additional serotypes: walrus calicivirus (WCV), reptile calicivirus (Cro-1), cetacean calicivirus (CCV Tur-1), Bovine calicivirus (BCV Bos-1) commonly referred to as the Tillamook calicivirus (TCV), and mink calicivirus (MCV) (Table 2). Although TCV and MCV have not been isolated from the

Table 1. Enteric Candidate Caliciviruses (Initial isolation in species)

Serotype	Species	Location	Year	Ref.
Norwalk virus	Human	United States	1972	32
Human calicivirus (HCV)	Human	United Kingdom	1976	32
Newbury agent of cattle		United Kingdom	1978	32,120
Porcine enteric calicivirus	Swine	United States, United Kingdom	1980	32,25
Amyelosis chronic stunt virus	Insect	United States	1981	52
Chicken calicivirus (CCV)	Chicken	United Kingdom	1981	32,121
Canine calicivirus (CaCV)	Dog	United States	1985	32,71
Non-A,Non-B hepatitis virus	Human	Asia, Africa, Mexico	1981-86	22

Table 2. Characterized Caliciviruses Demonstrating a Marine Presence (Initial isolation in species)

Serotype	Sources	Location	Year	Ref.
VESV 1-34	Swine	San Jose,CA	1934	31
VESV 101-43	Swine	San Francisco,CA	1943	26
VESV A_{48}	Swine	Fontana,CA	1948	55
VESV B_{51}	Swine	Davis,CA	1951	8
VESV C_{52}	Swine	San Francisco,CA	1952	8
VESV D_{53}	Swine	Riverside,CA	1953	5
VESV E_{54}	Swine	Warm Springs,CA	1954	6
VESV F_{55}	Swine	San Mateo,CA	1955	7
VESV G_{55}	Swine	San Mateo,CA	1955	7
VESV H_{54}	Swine	San Mateo,CA	1954	4
VESV I_{55}	Swine	San Mateo,CA	1955	4
VESV J_{56}	Swine	Secaucus,NJ	1956	49
VESV K_{56}	Swine	Secaucus,NJ	1956	49
SMSV-1	California sea lion	San Miguel Is,CA	1972	80,90
	Northern fur seal	St. Paul Is,AK	1972	90
SMSV-2	California sea lion	San Miguel Is,CA	1972	90
SMSV-3	California sea lion	San Miguel Is,CA	1972	80,90
SMSV-4	California sea lion	San Miguel Is,CA	1973	91
	Swine	Sonoma county,CA	1976	92
SMSV-5	Northern fur seal	St. Paul Is,AK	1973	91
	Animal feed	St. Paul Is,AK	1974	68
	Human	Corvallis,OR	1985	76
SMSV-6	California sea lion	San Miguel Is,CA	1975	79,108
	Northern fur seal	San Miguel Is,CA	1977	102,108
	Opaleye fish	San Nicolas Is,CA	1976	102
	Stellar sea lion	Rogue Reef,OR	1985	72
SMSV-7	Opaleye fish	San Nicolas Is,CA	1976	102,108
	N. Elephant seal	San Nicolas Is,CA	1976	102,108
	Sea lion liver fluke	San Diego,CA	1976	102,108
SMSV-8	Northern fur seal	St. Paul Is,AK	1975	104
SMSV-9	California sea lion	San Miguel Is,CA	1975	104,108
	Pacific dolphin	Honolulu,HI	1987	83
SMSV-10	Northern fur seal	St. Paul Is,AK	1977	104
SMSV-11	Northern fur seal	San Miguel Is,CA	1977	104
SMSV-12	California sea lion	San Miguel Is,CA	1977	104
	Northern fur seal	San Miguel Is,CA	1977	104
SMSV-13	California sea lion	Fort Cronkhite,CA	1984	19
SMSV-14	Stellar sea lion	Rogue Reef,OR	1987	83
	California sea lion	San Miguel Is,CA	1987	83
SMSV-15	California sea lion	San Miguel Is,CA	1988	83
SMSV-16	California sea lion	San Diego,CA	1988	83
Walrus (WCV)	Walrus	Chukchi sea	1977	93
	Walrus	Bering sea	1987	83
Cetacean(CCV Tur-1)	Atlantic dolphin	San Diego,CA	1979	107
	California sea lion	San Diego,CA	1979	107
Reptile (Cro-1)	Aruba Is rattlesnake	San Diego,CA	1978	82
	Rock rattlesnake	San Diego,CA	1978	82
	Eyelash viper	San Diego,CA	1978	82
	Bell's horned frog	San Diego,CA	1978	82

Table 2. Continued

Serotype	Sources	Location	Year	Ref.
Reptile (cont.)	California sea lion	San Miguel Is,CA	1986	83
	Northern fur seal	San Miguel Is,CA	1986	83
	Stellar sea lion	Rogue Reef,OR	1987	83
Mink (MCV)	Mink	Idaho	1977	37,53
Tillamook (TCV)	Cattle	Cloverdale,OR	1981	89

marine environment, serological evidence of marine calicivirus exposure (Table 3) indicates that they have a marine presence. With respect to the VESV's, one serotype has been isolated from a marine mammal (105) and, on the basis of serological testing, many other serotypes have been found to be present in the marine environment on the basis of serological testing (Table 3).

Included in the non-marine group are the strains of FCV, canine calicivirus, and primate calicivirus (PCV pan-1) (Table 4). Although these serotypes are considered non-marine in this study, their ocean activity remains unexplored. These agents have been associated with varied disease in several species. The FCV has been associated with respiratory disease (46) "limping" or lameness syndrome (61) and feline urolithiasis syndrome (39) in cats. In addition, FCV has been recovered from cases of glossitis in dogs (36, 37) and from canines experiencing diarrhea (38). The FCV causes intense viral cytopathic effect (CPE) in feline kidney cell lines (40) and is capable of establishing a carrier state in cats (115). Serological testing has revealed that several related strains of the FCV serotype exist and that cats worldwide have been exposed to this agent (46, 63).

Canine calicivirus has been recovered from dogs exhibiting vesicular genital lesions but does not appear to be related to the enteric candidate CaCV. This virus has been cultivated on feline kidney cells; however, serological testing has shown it to be unrelated to FCV (30).

The primate calicivirus has been recovered from non-human primates. The virus was isolated from a herpes-like lesion in a pygmy chimpanzee, gingival lesions in a spider monkey, oropharynx of a healthy silver leaf

Table 3. Marine Mammal Exposures to Caliciviruses Demonstrating a Marine Presence

Species	Serotype	Year	Reference
California sea lion	SMSV-1	1970-83	1,48,64,81,83,87
	SMSV-2	1970-76	1,48,64,81,87
	SMSV-4	1970-76	48,81,87
	SMSV-5	1970-86	48,81,83,87
	SMSV-6	1970-86	15,79
	SMSV-8	1977	83
	SMSV-10	1983-86	83
	SMSV-13	1977-86	19
	TCV	1983-84	17,76
	VESV	1972-76	87
Northern fur seal	SMSV-2	1970-76	64,87
	SMSV-5	1972-84	81,83,87
	SMSV-6	1975-77	79
	SMSV-10	1984	83
Stellar sea lion	SMSV-1	1976-85	11,28,83
	SMSV-2	1960-78	1,28
	SMSV-5	1976-85	11,28
	SMSV-6	1976-86	11,15,28,72
	SMSV-7	1985	11,15,28
	SMSV-8	1985	11,28
	SMSV-10	1976-85	11,28
	SMSV-13	1976-86	11,28
	TCV	1976-85	11,17,76
Dolphin	SMSV-1,SMSV-2, SMSV-5,SMSV-6, SMSV-12,SMSV-14, SMSV-15,WCV,MCV	1988	83
Walrus	SMSV-5,SMSV-8,WCV	1983	16
Bowhead whale	SMSV-5,SMSV-8, SMSV-10,VESV	1980	99
Gray whale	SMSV-2,SMSV-5,VESV	1968	1,87
Fin whale	SMSV-1,SMSV-5,VESV	1972	87
Sperm whale	SMSV-5,VESV	1972	87
Sei whale	SMSV-5,VESV	1972	87
N. Elephant seal	SMSV-4	1973	1
Hawaiian monk seal	VESV	1978	47

Table 4. Characterized Caliciviruses of Undetermined Marine Presence (Initial isolation in species)

Serotype	Species	Location	Year	Ref.
Feline calicivirus (FCV)	Cat	United States, Australia	1957	40
	Dog	United States	1981	36
Canine calicivirus	Dog	United States	1982	30
Primate (PCV Pan-1)	Pygmy chimpanzee	San Diego, CA	1978	103
	Spider monkey	San Diego, CA	1978	98
	Lowland gorilla	San Diego, CA	1978	98
	Silver leaf langur	San Diego, CA	1979	98
	Douc langur	San Diego, CA	1979	96

langur, spleen of a lowland gorilla that died of coccidiomycosis and a debilitated douc langur (96, 98, 103). Persistent virus shedding was detected for six months in one animal despite the presence of homologous neutralizing antibody. Contact transmission was believed to occur as pen-contact animals underwent seroconversion (98). This serotype was isolated and cultivated in vitro in African green monkey kidney (Vero) and porcine kidney (PK-15) cells.

The marine calicivirus group is composed of many serotypes and is the focus of this study. Previous work has shown that cattle possess type-specific, neutralizing, antibody against various serotypes of this group (19, 76, 83, 84). This study will attempt to quantify the prevalence and determine the distribution of exposure in cattle to several of these serotypes. To better understand the implications of exposure levels in cattle, a detailed review of the existing knowledge concerning these marine agents is necessary.

The VESV's were the first caliciviruses detected and made their appearance initially in swine. Since the SMSV serotypes used in this study are considered different serotypes of the eradicated VESV and that members of both groups are classified as indistinguishable from each other, a thorough review of VESV history and characteristics is useful.

Several detailed accounts of the history of VESV in swine exist (2, 34, 112). In retrospect, the first outbreak of VESV probably occurred on April 22, 1932 in Orange County, California, on a raw-garbage swine-feeding operation. The outbreak was characterized as a vesicular disease of low mortality and high morbidity, with lameness a frequent finding. Due to a recent history of foot-and-mouth disease (FMD) in California, this outbreak was assumed to be FMD. The affected swine

operations were quarantined, animals slaughtered with deep burial and premises thoroughly cleaned and disinfected in accordance with accepted FMD eradication guidelines. There was a lack of infective material for diagnostic confirmation due to the nature of FMD eradication procedures. Ten days after the outbreak started the disease was believed to have been eradicated.

Within 11 months, a second outbreak of vesicular disease in pigs occurred in San Diego County, California, on another garbage fed operation. During this outbreak, infective material was closely examined and FMD virus was not isolated. All eradication procedures employed previously were again instituted; however, strict quarantines were not imposed. A third outbreak occurred 15 months later that was 500 miles north of the second occurrence. This time, a diagnosis inconsistent with FMD was made and the disease was named vesicular exanthema (VE), (it is now believed that the first two outbreaks of vesicular disease were, in fact, VE). With this diagnosis, the Bureau of Animal Industry suspended its eradication efforts. State quarantines were imposed and affected animals were allowed to recover before going to slaughter.

Epidemiological investigations failed to uncover any identifiable physical links between the first three outbreaks. Meat, meat scraps, and other infective material from swine were not believed to play a role in the initiation of the first three outbreaks as all such material had been deeply buried or destroyed. The source of infection was considered therefore a mystery. When the disease was characterized as VE and not FMD, infected swine tissues became a possible link of transmission as recovered animals were allowed to be slaughtered and processed for food.

The third outbreak eventually spread to 37 swine ranches over a wide geographical area of the state. There were subsequent outbreaks in 1935 and 1936 followed by a three-year quiescent period. In 1939, vesicular disease outbreaks occurred in northern California swine herds and within six months one quarter of the herds in the state had VE involvement. The disease continued to be an ongoing problem with quarantine procedures proving ineffective in controlling spread.

Garbage feeding was soon recognized as an important mode in the transmission of VE. This conclusion was supported by the much higher incidence of VE found on raw-garbage farms as compared to grain-feeding operations and by the common link of garbage feeding to the first three VE outbreaks. However, it was a mystery why VE had been confined to California when raw-garbage feeding of swine was occurring in other states. The importance of this transmission route was recognized and attempts were made to require cooking of raw garbage fed to swine. The swine industry resisted and the practice of raw-garbage feeding continued.

In 1952, the first case of VE was reported outside California. The infection was traced to a herd in Cheyenne, Wyoming, where pigs had been fed raw garbage from a transcontinental train originating in California. The disease spread rapidly throughout the U.S. with 42 states becoming involved. Within two months after the spread of VE from California, the Bureau of Animal Industry declared a state of national emergency. The federal government embarked upon an eradication effort based on FMD procedures also requiring the cooking of raw garbage fed to swine. Imposed restrictions on animal movement forced raw-garbage cooking by closing interstate markets to swine products generated from raw garbage.

This placed increased economic pressures on swine producers and by 1953 a total of 46 states had adopted garbage-cooking laws. With these efforts, the incidence of VE declined with the last outbreaks occurring in New Jersey in 1956. On October 22, 1959, the U.S. Secretary of Agriculture announced the eradication of VE and, at that time, designated it a reportable exotic disease (2, 34, 112). The direct cost of VE eradication was set at $39,000,000 (13).

Through the entire history of VE, the disease was found in only two places outside the continental U.S. Outbreaks occurred in shipments of slaughter hogs received at the port of Honolulu, Hawaii in 1946 and in 1947 that originated from the U.S. mainland. Another outbreak occurred in 1955 on a swine farm in Hrafnarfjord, Iceland, where raw garbage from a nearby U.S. military base had been fed to swine (2).

During the relatively short course of VE in swine, 13 antigenic types were identified by cross-immunity animal inoculation and virus neutralization testing (Table 2). Four antigenically distinct types were collected during the outbreaks of 1933 and 1934 alone (31). These four serotypes were lost and their relationship to characterized serotypes is not known (2). Based on the typing of hundreds of isolates it is believed that, with the exception of VESV types J and K found in New Jersey, VESV B_{51} was the only serotype involved in outbreaks outside of California (35). Bankowski (2) suggested that mutation or genetic instability could explain the emergence of the large number of VESV serotypes found in California. New serotypes that seemingly arose "de novo" in recurrently-infected herds despite the thorough cooking of garbage supported this suggestion. Likewise, it was believed that the appearance of VESV J_{56} and VESV K_{56} types in New Jersey was due to a

rapidly mutating VESV B_{51} serotype (7, 35).

Vesicular Exanthema was characterized as a disease of variable morbidity, usually 30-80%, and low mortality especially in older animals (3, 34). Clinical disease in swine was variable and could be produced either by natural or experimental infection. Infection was characterized by Bankowski (2, 3) as beginning with a febrile response, typically 40-41.7°C, with subsequent formation of vesicles containing clear, straw-colored fluid. Vesicle formation occurred on the snout, any portion of the mouth, between the toes, on the sole of the feet or dewclaw, and on the udder and teats of nursing sows. Highly-stressed areas of the skin were frequently involved.

The virus was found to multiply in the malpighian layer of the epithelium causing hydropic degeneration and edema. This resulted in separation of the epidermis from the dermis and produced the typical vesicles observed. On a cellular level, infected cells ruptured allowing virus to infect adjacent tissue thus forming microvesicles which coalesced centrifugally to form larger macrovesicles (44). Upon rupture of the vesicles, raw ulcerated areas remained with a reduction in fever occurring. With milder strains, small vesicles formed with the epithelium frequently adhering to the ulcer. Temporary loss of appetite and lameness were observed until the vesicles resolved. With coronary band involvement, a dark line appeared on the hoof wall after recovery and was a good indicator of previous infection. Recovery usually occurred within five to seven days after vesiculation in uncomplicated cases (2, 3).

Other disease syndromes frequently associated with VESV infection were abortion and subsequent breeding difficulties, agalactia, diarrhea,

runting, myocarditis, and encephalitis (2, 44, 74). Pneumonia, septicemia and hoof wall separation with resultant hoof loss were infrequent findings (2). Unthrifty and non-profitable animals caused primarily by agalactia and the runting syndrome in young pigs were responsible for the major economic losses felt by the swine industry (75).

The onset of disease after exposure to VESV was found to be quite variable. Experimental inoculation in swine by scarification of the snout or intradermal injection demonstrated that the incubation period for VE was typically 12-72 hours. After this period, primary vesicles at the point of inoculation appeared. Within two more days, hematogenous spread generally occurred producing secondary lesions at various locations on the body (2, 31, 44). The incubation period in field situations was 24-72 hours for most strains, with milder types taking up to 10 days to produce lesions from direct contact exposure. Incubation periods in swine inoculated by subcutaneous, intravenous, and oral routes were generally long ranging up to 12 days (2).

Virus doses required to produce infection vary with the route of inoculation. For example, it was found that the equivalent of 10-100 and 100-1,000 intradermal doses were required to achieve infection in swine via the intravenous and oral routes respectively (58). Inoculation by scarification of the snout and application of a paste consisting of ground vesicular tissue proved the most successful route of inoculation in experimental studies (7).

The pathogenicity of VESV serotypes was not uniform. Preliminary work with the four "lost serotypes" of VESV demonstrated differences in their ability to produce severe disease (31). Other investigators noted

that pathogenicity was unrelated to serotype and that morbidity rates were independent of disease severity (6). Experimental work later found that the pathogenicity of each serotype was dependent upon the particular strain involved (3). It was also found that the pathogenicity of a serotype is increased with repeated passage in swine (6). With regard to the pathogenicity character of VE outbreaks, milder strains of VESV tended to be recovered as the disease neared eradication. As an example, the strains of VESV J_{56} and VESV K_{56} isolated in 1956 caused only mild disease in swine in comparison to serotypes involved in earlier outbreaks. Interestingly, the presence of bacterial contaminants during infection with these two types was found to increase the severity of disease (49).

The pathogenicity of VESV serotypes is related to the plaque size produced in tissue culture. In vitro, the VESV E_{54} serotype produced several plaque sizes when cultured on swine kidney cells (57, 114). In vivo studies with swine using plaque-purified virus demonstrated that small-plaque sizes were of low virulence and larger types were capable of producing severe disease. The small-plaque variants were found to be more cell associated, easier to adsorb to cells, not as cytolytic and somewhat less stable than the large-plaque types. Further, a predominance of large-plaque variants were recovered from vesicular lesions produced by inoculation of swine with small-plaque types (57). Study of this phenomenon in swine and in canine and porcine cell lines using six different plaque types of VESV E_{54} revealed that plaque type mutations occur in stepwise progression from small-to-large. It was suggested that this mutation process logically favors virus survival (114).

Transmission of VE has been found to occur through several different routes. The feeding of raw garbage containing infective meat scraps was probably the most important. Historically, a switch in transmission was thought to occur after 1936. The first outbreaks appeared to be point source introductions having a seasonal occurrence and were effectively controlled by quarantine measure. Subsequent outbreaks seemed to switch to a swine to swine transmission cycle mediated by raw garbage feeding as these outbreaks were uncontrollable and occurred throughout the year (78). All tissues of VESV infected swine have been shown to be infective to other swine for a short period before and several days after vesicle formation in experimental feeding trials (56, 58). In addition, raw-garbage decomposition may have enhanced VESV infectivity as the process produces cysteine hydrochloride, which is very effective in reactivating the virus (56).

Raw-garbage cooking was largely responsible for the decline of VE however some outbreaks continued to occur despite these efforts (6). Adequate cooking was an important factor as examination of several types of cooking equipment revealed that grinding and mixing were of paramount importance to VESV inactivation (18). Variability in heat sensitivities between serotypes also was observed (2).

Several direct-contact transmission studies have been conducted. From this work, virus shedding was shown to occur shortly before and several days after vesiculation (58). Virus was found to be shed in feces, urine, and nasal and oral secretions (44). Vesicular lesions were probably the most important source of virus as they were laden with large numbers of infective virus (3).

Indirect transmission from feed and water contamination and fomites

was shown to be important. For example, transmission of VE occurred when susceptible swine were placed in pens three days after removal of infected animals (58). A similar attempt at transmission after seven days failed to produce infection (6). Biting insects, such as the body louse of swine, were shown to be infective and may have been mechanical vectors (3). Transmission through intact hair follicles was attempted but not achieved (44).

Subclinical infections of VE have been demonstrated in swine. Transmission involving this disease state may have been responsible for the occurrence of repeated outbreaks of VE on individual farms. For instance, some cooked-garbage operations that routinely received replacement animals experienced repeated outbreaks involving single serotypes (6, 58).

The VESV's were found to be relatively stable and could be stored for long periods of time. Some VESV types remained viable for up to six weeks at room temperature (56). In addition, the virus was found to be infective after storage for 10 years at 4°C (4). Resistance to non-ionic detergents, iodine-based disinfectants and partial resistance to proteolytic enzymes was demonstrated (32). The virus was found to be susceptible to phenolic disinfectants, hypochlorite, 2% lye, and aldehydes with a 2% lye solution routinely used as a disinfectant during the VE outbreaks (2, 32).

Vesicular Exanthema in swine was clinically indistinguishable from several other vesicular diseases but could be differentiated by host-susceptibility testing. The VESV's were capable of causing mild disease in horses; however, reportedly was not capable of infecting guinea pigs or cattle. In contrast, the FMD virus is capable of infecting cattle

and guinea pigs but not horses. Vesicular stomatitis (VS) virus produces lesions in all three species. This method of disease differentiation was used commonly during the VE outbreaks (31, 113). Swine vesicular disease (SVD) virus, discovered after VE eradication, causes a disease syndrome in swine that is clinically indistinguishable from VE but does not affect horses, cattle, and guinea pigs (13).

Due to the cost of testing and time involved with host-susceptibility testing, other diagnostic tests were developed. Tissue culture methods utilizing swine kidney cells were employed. This made disease detection less expensive and faster but also less sensitive than animal inoculation (4). With utilizing the complement fixation test, not only could the virus of VE be differentiated from the viruses of other vesicular diseases but individual VESV serotypes could be identified (8, 35). This is now the standard test used for detection and differentiation of the four vesicular diseases of swine (50). Like the complement fixation test, the serum neutralization test was found capable of detecting individual serotypes of VESV as well as differentiating the vesicular diseases (49). Cross-reactivity of serotypes was observed on the agar-gel diffusion test when utilizing hyperimmune VESV antisera (35). With the use of convalescent sera this test was capable of serotype specificity (66).

Host-susceptibility studies using observed clinical syndromes as the basis for evidence of infection have been carried out in several species. Species found uniformly resistant to VESV infection include cattle (2, 31, 49, 55, 113), guinea pigs (4, 31, 55, 113), chickens and chick embryos (2, 49, 55), goats, sheep, mice, rats, rabbits and the hedgehog (31, 55), mink (66) and the armadillo (118). Species

susceptible to VESV infection in addition to swine include horses (2, 31, 49, 55, 113), dogs and hamsters (2, 55, 66), primates (92) and both otarid and phocid seals (43, 45). These species were found to be susceptible to varying degrees depending upon the serotype used. The clinical syndrome observed in horses was characterized by the formation of small lesions at injection sites with no extension and a slight rise in temperature (31). In vitro, susceptibility testing with the VESV's revealed that CPE was produced routinely in swine kidney cell lines. Other cell lines inoculated but found negative for CPE included bovine, mice, monkey and human (49). More recent work with the VESV A_{48} serotype demonstrated that growth occurred in canine, feline and human cell lines (74, 88).

Cross-immunity testing was the basis for VESV serotype classification. Initial testing in 1937 revealed that no relationship existed between the viruses of VE and FMD; however, limited cross-immunity was reported with the VS virus (31). The serum-neutralization test correlated well with cross-immunity VESV typing and could be performed with reasonable speed and ease (2, 43, 44, 49, 56). Neutralizing antibody has been detected as early as 3 to 12 days post infection with a peak in titer usually reached by 21-28 days. The rise in neutralizing antibody was shown to correlate well with viral clearance in vivo (44). In limited studies, it was reported that neutralizing antibody dropped to undetectable levels by 50 days post-inoculation in swine (45). Other work shows that detectable, neutralizing, titers can persist at high levels for up to 7 months (50). Homologous virus challenge in swine re-inoculated six months to a year after initial exposure did not produce visible disease (2, 56). Vaccine

development has been attempted with a certain degree of success; however, the protection conferred was serotype specific (56). The belief that VESV's undergo rapid antigenic mutation combined with the multitude of existing antigenic types would have made vaccine development a difficult task (90).

The SMSV's and other marine caliciviruses are indistinguishable from the VESV, differing only with respect to the species of original isolation. Typing of the SMSV's is based on the serum-neutralization test with new serotypes defined when 20 antibody units of known hyperimmune serum fail to neutralize 100 infective doses of challenge virus (74, 77). The first isolations of SMSV were made in 1972 with new serotypes presently being isolated and characterized (Table 2). The first known appearance of the SMSV's occurred during an investigation of the causes of reproductive failure in pinnipeds. Over a two year period, this investigation yielded four serotypes that were indistinguishable from the VESV's on the basis of physicochemical and morphological properties and animal infectivity profiles (48, 80, 90, 91).

San Miguel sea lion virus type one (SMSV-1) was recovered from rectal swabs of two California sea lions (Zalophus californianus) that had recently aborted on San Miguel Island, CA. This serotype also was recovered from the nose of an emaciated northern fur seal pup (Callorhinus ursinus) on St. Paul Island, AK in the same year. A second serotype, SMSV-2, was isolated in 1972 from the throat and rectum of a California sea lion aborting 30-60 days before term on San Miguel Island. A third serotype, SMSV-3, was recovered in the same year from the nose of a fetus aborted by one of the sea lions yielding the SMSV-1

isolate. This serotype cross-reacted with antiserum to the first two SMSV types and was thought, therefore, to be a mixed infection of SMSV-1 and SMSV-2 (13, 90).

The SMSV-4 and SMSV-5 serotypes have been recovered from several different species. The SMSV-4 type was isolated first from the throat of two aborted California sea lion fetuses on San Miguel Island in 1973 (91). This serotype was isolated again in 1976 from throat and rectal swabs of three, garbage-fed, swine exhibiting lameness disease from a Brucella infected herd in Sonoma County, CA (92, 97). SMSV-5 was recovered initially from vesicular lesions on the flipper of a northern fur seal in 1973 on St. Paul Island, AK (91). This same serotype was isolated from mink food comprised of northern fur seal carcasses processed during the 1974 annual fur seal harvest in Alaska (68). In addition, isolation of SMSV-5 was made from blisters on the hands and feet of a human experiencing flu-like symptoms in 1985. In this case, infection was thought to have occurred from accidental exposure of a laboratory researcher working with the SMSV's (76).

The SMSV-6 serotype was recovered initially from vesicular lesions of two California sea lion pups on San Miguel Island in 1975. A throat swab of a northern fur seal sampled on San Miguel Island in 1977 also yielded SMSV-6 (79, 102, 108). This same serotype was recovered from the spleen of an opaleye fish (Girella nigricans) collected from tidal pools on San Nicolas Island, CA in 1976 (102) and in 1985 from a rectal swab of a stellar sea lion pup (Eumetopias jubatus) on Rogue Reef, OR (72).

The SMSV-7 serotype was isolated in 1976 from throat and rectal swabs of four, clinically normal, northern elephant seal pups (Mirounga

agustirostris) on San Miguel Island. In addition, ground visceral tissues of two opaleye fish from the same sampling that yielded the SMSV-6 isolate and a whole macerated liver fluke (Zalophatrema sp.) taken from a California sea lion that died of verminous pneumonia in San Diego, CA, in 1976 yielded SMSV-7 serotype (102, 108).

The next five serotypes, SMSV-8, 9, 10, 11, and 12 were recovered over a two-year period from 1975-1977 (104, 108). The SMSV-8 and SMSV-10 serotypes were isolated from vesicular lesions of one and six northern fur seals respectively, during the annual harvests on the Pribilof Islands, AK. The SMSV-9 serotype was isolated initially from the throat of a California sea lion pup on San Miguel Island and subsequently in 1987 from a Pacific bottlenose dolphin (Tursiops gillii) in Hawaii (83). The SMSV-11 serotype was recovered from throat and rectal swabs of two northern fur seal pups both from San Miguel Island. The SMSV-12 serotype was isolated from throat and rectal swabs of a California sea lion pup and northern fur seal pup both sampled on San Miguel Island.

In 1984, a vesicular disease outbreak occurred among California sea lions received at the California Marine Mammal Center in Fort Cronkhite, CA. Vesicular lesions were observed on the flippers of 39 out of 250 sea lions with a new serotype, SMSV-13, being isolated from the vesicles (19).

Recently, three serotypes of SMSV have been recovered from the marine environment and have been designated as SMSV-14, SMSV-15 and SMSV-16. In 1987, the SMSV-14 serotype was recovered from clinically normal stellar sea lion pups on Rogue Reef, OR, and from California sea lion pups on San Miguel Island. The SMSV-15 and SMSV-16 serotypes were

isolated from California sea lions in 1988 with SMSV-16 recovered during an outbreak of diarrhea at a marine mammal facility (83).

The remaining marine serotypes are not designated as SMSV's but instead have been named after the species or geographical location of original isolation.

The walrus calicivirus was recovered as three separate isolates from fecal samples collected on pack ice in the south central Chukchi Sea in 1977 (93). This type was again recovered from walrus sampled in the Bering Sea in 1987 (83).

Cetacean calicivirus was initially recovered in 1977 from a tattoo pox lesion that developed into a vesicle on an Atlantic bottlenose dolphin (Tursiops truncatus) at a holding facility in San Diego, CA. The disease appeared to spread to a clinically normal sea lion at the same facility and to another dolphin which subsequently developed vesicular disease at a nearby facility (107).

The mink calicivirus was obtained in 1977 from pharyngeal and rectal swabs of clinically normal mink. These animals were housed near other mink that had recently died of hemorrhagic pneumonia (pseudomonas pneumonia) on two commercial mink farms in Idaho (37, 53).

Reptile calicivirus was recovered first in 1978 from various reptiles and amphibians including 12 Aruba Island rattlesnakes, a rock rattlesnake, eyelash viper, and 2 Bell's horned frogs housed at the San Diego Zoo (83). The virus was isolated from various tissues and rectal swabs of both healthy and diseased animals. Although many animals were exhibiting severe disease, the causal relationship of between virus isolation and disease was not resolved. Reptile calicivirus was found in the marine environment in 1986 when it was recovered from both a

California sea lion and northern fur seal sampled on San Miguel Island. This serotype was subsequently recovered from a rectal swab of .a stellar sea lion sampled on Rogue Reef, OR, in 1987 (83).

Tillamook calicivirus was isolated in 1981 from three calves in a dairy herd with a history of persistent, calf respiratory disease. The virus was isolated from rectal and throat swabs with re-isolation accomplished three weeks later in one calf. Although the virus was found in calves with respiratory disease, the causal relationship between virus recovery and calf pneumonia was unclear (89).

From the virus recoveries discussed above, there is strong evidence that the SMSV's and other marine caliciviruses do, with natural infection, cause a vesicular lesions in California sea lions, northern fur seals, dolphins and man. In 1978, an attempt was made to quantitate the number of animals exhibiting vesicles during the annual harvest of northern fur seals on the Pribilof Islands, AK. Of 25,000 seals examined, .1% demonstrated visible lesions (43). From an earlier study done in 1974, it was estimated that 2% of the fur seals killed during harvest had vesicular lesions (68). Lesions from natural infection in marine mammals were characterized as being up to three centimeters in diameter, filled with clear fluid or pus, and located on both the dorsal and ventral surface of flippers. The vesicles usually ruptured leaving an area of eroded epithelium. Regression of vesicles without erosion occurred in some cases (77).

Other clinical syndromes found to be associated with natural infection included abortion and diarrhea in California sea lions, hemorrhagic pneumonia in mink and persistent respiratory disease in calves. In other cases of natural infection, these viruses have been

isolated from clinically normal animals. This may represent asymptomatic, persistently infected, animals or animals experiencing self-limiting subclinical infection. It is noteworthy that several SMSV serotypes have been isolated from aborting sea lions and aborted fetuses especially when considering that the SMSV's are indistinguishable from the VESV's and that the VESV's were frequently associated with abortion in swine. The possible causal relationship of SMSV infection to abortion in marine mammals remains unclear (13, 90, 91).

Experimental infectivity studies with the SMSV's and other marine caliciviruses have yielded insights into susceptible hosts and resultant diseases produced by these agents. With respect to marine mammals, northern fur seals and harp seals (Phoca groenlandica) have been experimentally inoculated with SMSV-2. Resultant disease ranged from small-plaque lesions at intradermal inoculation sites to inapparent infection (43, 45).

Swine have been inoculated experimentally with several marine calicivirus serotypes. Intradermal exposure to SMSV-2 has produced mild vesicular disease in swine. In addition, SMSV-2 was recovered from brain tissue indicating that these agents may cause encephalitis (44, 74). Swine fed ground seal carcasses exhibiting SMSV-5 induced lesions developed a severe vesicular disease with virus being isolated from both blood samples and rectal swabs. Clinical disease was observed two days after consumption of contaminated meat and was characterized by severe depression, loss of appetite, lameness, fever of 41°C, diarrhea and loss of weight in addition to the formation of vesicles on the feet and snout (117). Comparison of virus morphology before and after transmission of SMSV from pinnipeds to swine revealed that no significant changes had

occurred (24). Swine inoculated experimentally with SMSV-7 developed clinical vesicular exanthema with the formation of secondary vesicular lesions. Horizontal spread to pen-contact animals also was observed (102). Swine infectivity studies with several SMSV serotypes demonstrated that these agents have hepatotrophisms. Walrus calicivirus for instance, produced hepatocellular degeneration from intradermal, oral and pen-contact exposure in swine. Pneumonitis and enteritis were observed also; however, a causal relationship was not established (83, 86). Swine inoculated with TCV developed lesions only at injection sites, however, extension of these lesions occurred. Even in the absence of severe vesicular disease, this serotype produced a more severe disease in swine than many of the VESV serotypes (89). Experimental inoculation of swine with the reptile calicivirus serotype failed to produce visible lesions; however, seroconversion occurred in all subjects including pen-contact animals (82).

Calves have been inoculated experimentally with SMSV-5, SMSV-13 and TCV. The SMSV-5 serotype failed to produce visible lesions; however, SMSV-13 produced severe lesions at the site of intradermal inoculation and secondary vesicular lesions by the fourth day post-inoculation. Spread to a pen-contact calf also occurred (76). Cattle inoculated with TCV developed lesions at intradermal injection sites without secondary vesicle formation. In a comparative disease study, this serotype was shown to produce less severe disease in cattle than in swine (89).

Experimental inoculation in horses with SMSV types 1-5 caused erosive tracts at the sites of intradermal inoculation. A temperature rise was detected; however, none of the lesions spread by extension

(119).

Mink fed ground fur seal carcasses exhibiting SMSV-5 induced lesions developed an inapparent infection with virus being isolated from blood samples and rectal swabs. (117). Mink kittens inoculated with MCV did not develop clinical disease. Even so, it is thought that this MCV may predispose mink to severe cases of hemorrhagic pneumonia as it was isolated during outbreaks of this disease (53).

The African green monkey (Cercopithecus aethiops) has been exposed experimentally to SMSV-4 and SMSV-5. Both serotypes produced lesions at intradermal injection sites which spread by extension. A rise in temperature occurred within 72 hours and lesions regressed and healed by the seventh day post-inoculation (92).

Experimental inoculation of two adult rattlesnakes with reptile calicivirus has been attempted. Both snakes failed to develop recognizable calicivirus-induced lesions (82).

Opaleye fish held at a temperature of 15°C have been inoculated experimentally with SMSV-5 by oral and intraperitoneal routes. Generalized infection resulted; however, no clinical symptoms were observed (106).

Susceptibility testing of common laboratory animals such as rabbits, hamsters, rats, mice and guinea pigs to SMSV-4 and SMSV-5 has been carried out. None of the species tested demonstrated detectable infection (88).

In vitro susceptibility to the SMSV's is broad with host cells from phylogenetically diverse species supporting viral replication. The SMSV types 1, 2, 4 and 5 have been shown to grow well in feline, canine, porcine, human, and primate cell lines. In addition, these serotypes

demonstrated variable growth in herbivore and rodent cell lines. No replication was observed in the avian and marine mammal cell lines tested (80, 88). In another study, SMSV-2 demonstrated the ability to replicate in a whale kidney cell line (1). The mink, walrus, Tillamook and reptile caliciviruses have demonstrated the ability to replicate in Vero cells (37, 53, 82, 89, 93). The ability of some SMSV's but not others to infect specific cell lines and the adaptability of some serotypes to certain cell lines have been reported (88). With respect to adaptability, SMSV propagated in vitro has been reported to produce less severe disease in swine than virus propagated in vivo (117). In tissue culture, many of the SMSV's have produced high titers and CPE within 8-10 hours post-inoculation. Other types have been shown to replicate to high titer without causing CPE (95). Calicivirus CPE in Vero cells has been characterized at an ultrastructural level. Cells were shown to undergo a loss of microvilli and pseudopodia and become rough and shrunken as viral infection progressed (94).

Pathogenicity of the SMSV's is variable and dependent upon serotype. Variability between SMSV serotypes with respect to the production of disease in swine has been noted (44, 74, 76, 80, 89). As an example, severe vesicular disease was produced by SMSV-13 but only mild disease resulted from SMSV-2 exposure in swine. In the marine environment the same variability occurs, as some serotypes were obtained from severely diseased animals yet other types were recovered from animals with no observable disease. Just as with VESV, the plaque size produced on tissue culture for any given serotype of SMSV is variable (80). It has been shown that purified large-plaque variants of SMSV serotypes are less cell associated and may be more virulent than minute-

plaque variants. In addition, plaque size has been compared to poly C length found upon oligonucleotide fingerprinting of SMSV RNA. Large-plaque variants were shown to possess longer Poly C segments than the minute variants. It has been suggested that the poly C length of any given serotype could be used as a indicator of its pathogenicity (42, 62).

There are several routes by which the SMSV's gain entry into susceptible species. Experimentally, swine have been infected with SMSV-5 by intradermal, intranasal and oral routes (117).

Several examples of marine calicivirus transmission have been observed. The cetacean calicivirus reportedly was shared between a California sea lion and dolphins at two marine mammal holding facilities. Common water and animal handler contact were suggested mechanisms for spread of the virus (107). Fecal contamination and direct contact from animal handlers were implicated in the transmission of the reptile serotype at the San Diego Zoo. The virus source to reptiles and amphibians in this occurrence was never found (82). Direct contact transmission may have been responsible for a "blisters on the eyes" syndrome of suspected calicivirus etiology that was experienced by a biologist after working with fur seals exhibiting vesicular lesions (77). Transmission by direct contact from ruptured virus laden vesicles has been shown to be an important route of exposure in several species (76, 89). Direct contact transmission has been reported to occur in pen contact animals used in experimental infectivity studies (82, 102).

Transmission through the consumption of contaminated feedstuffs has been suggested for the observed widespread exposure of mink to MCV. It is known that whole northern fur seal carcasses produced from the from

the annual Pribilof Islands harvest have, since 1963, been ground up as "sealburger" and shipped frozen to mink farms in the U.S. Experimental transmission studies have shown that this "sealburger" may be infective for both swine and mink when consumed (68, 117). It is interesting to note that it is common practice for mink producers to use swine to scavenge feed scraps that drop through mink cages. This practice certainly enhances the potential spread of MCV to other species (53, 68, 78).

Experimental transmission of SMSV-5 to northern fur seals from infected opaleye fish held at 15°C has been accomplished. The fur seal pups, upon consuming the infected fish, developed characteristic vesicular disease. Adding another step to the transmission cycle, the sea lion lung worm (Parafilaroides decorus) was inoculated experimentally with SMSV-5 and was found to be capable of transmitting the virus to opaleye fish. The infected fish subsequently produced SMSV-5 induced lesions in fur seals when consumed. This study confirmed that a nematode-teleost-marine mammal cycle for SMSV-5 could exist. The entire sequence of events, from lung worm inoculation to fur seal infection, covered a span of 54 days (101). It was theorized that a single opaleye fish could transmit several serotypes of calicivirus to predator marine mammals by encysting several lung worm larvae infected with various SMSV serotypes (15, 73).

Vertical transmission within a species may occur as SMSV-2 has been isolated from the testicle of an experimentally infected northern fur seal (43). Transmission of virus through exposure to contaminated water has been suggested as SMSV-5 has been found to have good survivability in sea water held at 15°C (106).

Marine calicivirus carrier states in several species have been described. This disease condition has been reported in northern fur seals infected with SMSV-2 and SMSV-5 (43, 101), mink inoculated with SMSV-5 (53) and calves infected with TCV (89). Shedding of Tillamook calicivirus by calves was observed for 45 days at which time the study was terminated.

The immunological response to the SMSV's and other marine caliciviruses is type specific and occurs soon after exposure. Neutralizing antibody generally appears within 3 to 5 days post inoculation (43, 45). Experimental SMSV infection in monkeys produced a significant antibody response on the second day post-inoculation with peak levels being reached by the fourth week. In this case, antibody was maintained at a high titer for 90 days at which time the study was terminated (92). Neutralizing titers to the SMSV's in naturally or experimentally infected species have been shown to be low with a typical range of 1:10 to 1:640 (31, 76, 79, 82, 87, 93). Some titers as high as 1:2560 in marine populations have been recorded (81). In contrast, calves naturally infected and shedding TCV have demonstrated titers no higher than 1:10 (89).

Several immunological tests are used for the detection of SMSV and its antibody. The complement fixation test, utilizing hyperimmune sera of polyvalent activity, is used currently as the official assay for detection of VESV and SMSV antigens in tissues and vesicular fluid. The virus neutralization test is used for VESV and SMSV serotype differentiation as well as for detection of type specific antibody. The specificity of the neutralization test in detecting antibody is evident as high antibody titers to one serotype in a serum sample frequently are

observed without cross-reaction to other SMSV serotypes (77, 81, 87). The agar-gel diffusion test has been applied to SMSV virus and antibody detection with antigenic cross-reactivity to VESV and other SMSV serotypes common. A single protein antigen is believed to be responsible for this cross-reactivity (50). The indirect immunofluorescence test has been found to be useful in calicivirus detection however variable antigenic cross-reactivity between SMSV and VESV serotypes occurs. This assay detects group antigens on cell surfaces in contrast to the virus neutralization test which detects free viral antigens. The indirect immunofluorescence test also has been utilized as an antibody assay and is capable of detecting antibody as early as 5-7 days post-inoculation and for a period of up to 5 months (50, 116). The radioimmune precipitation test has been utilized for calicivirus detection with antigenic cross-reactivity occurring between the VESV and the SMSV serotypes (110).

Exposure to marine caliciviruses, as determined by virus neutralization testing, is widespread among both marine and terrestrial populations (Tables 3 and 5). From these tables, it is apparent that many species have been exposed to multiple serotypes and that terrestrial animals possess antibody to many of the marine serotypes. Conversely, the marine environment appears to have an ongoing involvement with the VESV's which originally were considered to be of terrestrial origin. In addition, some marine mammal species that are essentially isolated from one another in the marine environment exhibit exposure to many of the same serotypes. From this information, it would appear that the land-sea interface is not a significant barrier to calicivirus movement. It should be noted that the accumulated exposure

Table 5. Terrestrial Animal Exposures to Caliciviruses Demonstrating a Marine Presence

Species	Serotype	Location	Year	Reference
Feral swine	SMSV-1,SMSV-2,	Santa Cruz Is, CA	1973	64,81,87
	SMSV-5		1973	81,87
	VESV A_{48},VESV C_{52}		1973	87
	VESV D_{53},VESV E_{54}			
	VESV G_{55},VESV I_{55}			
	VESV K_{56}			
Farm swine	SMSV, VESV A_{48}	California, Idaho, Utah	1976	73,75,100
Feral donkey	SMSV-2,VESV I_{55}	San Miguel Is, CA	1976	87
Gray fox	SMSV-2,SMSV-5	Santa Cruz Is, CA	1973	65
Mink	MCV	Idaho, Michigan, Montana, New York, Oregon, Washington, Wisconsin, Japan	1977	53
Musk ox	SMSV-5	San Francisco, CA	1987	83,85
Bison	SMSV	Santa Catalina Is, CA	1977	73,85
Cattle	SMSV-5,SMSV-13	Oregon	1984-85	19,76,84
Sheep	SMSV-2	Channel Is, CA	1973	81
	SMSV-13	Oregon	1985	83,84
Human	SMSV-4,SMSV-5	California	1978	92

data is based upon the examination of a small number of sera for the presence of type specific antibody.

The disease syndromes produced by SMSV and VESV infection have been compared in several species. For example, SMSV induced lesions found in northern fur seals have been characterized as indistinguishable from lesions produced in swine infected with VESV (43). In other studies, swine experimentally infected with 6 serotypes of SMSV developed vesicular disease that could not be differentiated from VE (73, 74, 75, 89). Furthermore, comparative disease studies have revealed that very similar lesions are produced in monkeys (92), horses (119), northern fur seals and harp seals (43, 45), various laboratory animals and swine when inoculated with either VESV or SMSV (80). The variation in disease severity produced by members of the SMSV and VESV groups appears to be as great as the differences in severity produced by individual serotypes within each group. Considering this, the SMSV's have been classified as indistinguishable from the VESV's in their ability to cause clinical disease (73).

The physical characteristics of SMSV have been compared to those of VESV by examining a few serotypes representing each group. In these comparisons, many similarities have been found (80, 111). Biochemical comparisons have revealed that the amino acid composition of the protein coat of a few members of both groups is very similar (109). RNA homology and tryptic peptide map studies also have demonstrated the relativeness of the VESV and SMSV groups. In addition, the RNA base composition of SMSV and VESV was found to be similar (27). It should be noted that the differences between serotypes within each group were not compared. Therefore it is not known whether greater or less variation

exists between serotypes in a group in comparison to differences found between the VESV and SMSV groups.

Some significant differences have been observed between SMSV and VESV when using one serotype to represent each group. In one study, VESV A_{48} was reported to have a greater resistance to chemical disinfectants than SMSV-2 (21). Others have reported that VESV A_{48} and SMSV-2 have different in vitro growth patterns on feline embryo cell lines (27). In addition, variation in the ability of several serotypes of SMSV and VESV to grow on Vero and swine testicle cells has been observed (50). Again, since the differences between all serotypes within the VESV and SMSV groups were not investigated, it is not known whether the variations between serotypes of each group are greater or less than those observed between the two groups.

Antigenic relatedness studies concerning the SMSV's and VESV's have been carried out. Immunoelectron microscopy (IEM) was utilized for some of these comparisons and has revealed that most of the VESV's and SMSV's are antigenically related to each other. In addition, two types of VESV were found to cross-react with FCV but not with each other. One of these two types reacted with the SMSV's but not with other VESV's (108). Another study utilizing IEM techniques revealed that SMSV-6 had some partial antigenic relatedness to FCV (79). Comparisons using the serum neutralization test revealed that the antigenic relatedness between the VESV and SMSV groups was as great as the relatedness between serotypes within each group (21, 78, 104) From this work, the basis for the distinction between VESV and SMSV serotypes is not well defined.

Studies into the relationships among members of the SMSV group have been carried out. Oligonucleotide fingerprinting has revealed that

serotype relatedness may be associated with host-spectrum characteristics. For example, it was found that serotypes isolated from similar hosts, such as is the case with SMSV-6 and SMSV-7, had more oligonucleotides in common than serotypes isolated from dissimilar hosts such as the reptile and mink caliciviruses (42).

Summarizing the available information, the marine caliciviruses are capable of producing similar disease in a wide variety of hosts including many species of domestic livestock and man. These viruses appear to be widespread in the marine environment and also demonstrate a presence in terrestrial animals. Evidence from serological surveys and experimental transmission studies indicate that the ocean/land interface is not an effective barrier to movement of these viruses. Considering the widespread exposure and disease causing potential of these viruses, an assessment of the prevalence and distribution in domestic livestock is desirable. Consequently, this study is designed to determine the exposure of selected U.S. cattle to several of the marine caliciviruses.

MATERIALS AND METHODS

Survey sample selection

Serum samples from 1,046 Kansas cattle were selected in a random fashion from groups of blood samples submitted to the State/Federal Brucellosis Laboratory, Topeka, KS, as part of routine Brucellosis screening of market and farm cattle from March to September of 1988. The sample size chosen was determined, on a statistical basis, to be the number of samples required to provide a 95% confidence limit and 3% error bound for an unknown population prevalence of calicivirus exposure (33). To provide an estimate of the SMSV-5 antibody prevalence, a pilot study was carried out by testing 75 serum samples from cattle of three geographically separated Kansas counties. The results confirmed that there was a significant presence of SMSV-5 antibody. The number of cattle sera selected to represent each county was based on 1988 Kansas cattle census information[1]. Using this approach, approximately .02% of the stock cattle in each county were tested. A total of 523 farms were involved in the study with two samples selected in a random manner from each premise. Since the sera of market cattle were included in this study, efforts were made to screen out samples from trader cattle not originating from the county represented. The blood samples were prepared for shipment by removal of the clot and were frozen prior to transport to the testing laboratory.

Each of the 1,046 sera were examined for neutralizing antibody to SMSV-5, 7, 14 and the Tillamook serotype. In addition, a subsample

[1]Cattle County Estimates: 1988. Kansas Agricultural Statistics, Kansas State Board of Agriculture/U.S. Department of Agriculture, Topeka, Kansas.

comprised of 158 sera from 16 counties was constructed. The 16 counties were selected on a random basis and the number of sera representing each county was the same as in the statewide sample. All sera in the subsample were examined for neutralizing antibody to the SMSV types; 1, 8, and 13.

The age, breed and sex characteristics of the statewide sample are illustrated in Figures 1, 2 and 3 respectively. The number of animals sampled from each county for the statewide survey is shown in Figure 4 and the distribution of the counties included in the subsample is illustrated in Figure 5.

Virus source

Plaque purified virus stocks of SMSV-1, 5, 7, 8, 13, 14 and the Tillamook serotype were obtained from the virus repository at the Central Laboratory for Calicivirus Studies, Oregon State University, College of Veterinary Medicine, Corvallis, OR. Each serotype had been serially passaged in Vero cells prior to utilization in this study. Working virus stocks were produced by adsorbing a 1:100 dilution of each virus to a confluent monolayer of low passage Vero cells grown in 75cm^2 tissue culture flasks (Corning). Eagle's Minimum Essential Medium (MEM, Gibco) was used as a diluent and was supplemented with Non-essential Amino Acids (Gibco): .1 mM/ml MEM, Sodium Pyruvate (Gibco): 1mM/ml MEM, L-Glutamine (Gibco): 20ug/ml MEM, gentamicin (Gibco): 2mM/ml MEM, and Penicillin-Streptomycin solution (Gibco); Penicillin base: 50 units/ml MEM and Streptomycin base: 50ug/ml MEM. The infected cells were maintained in MEM containing 3% defined and filtered fetal bovine serum (FBS, Hyclone). Fetal bovine serum used in all laboratory procedures

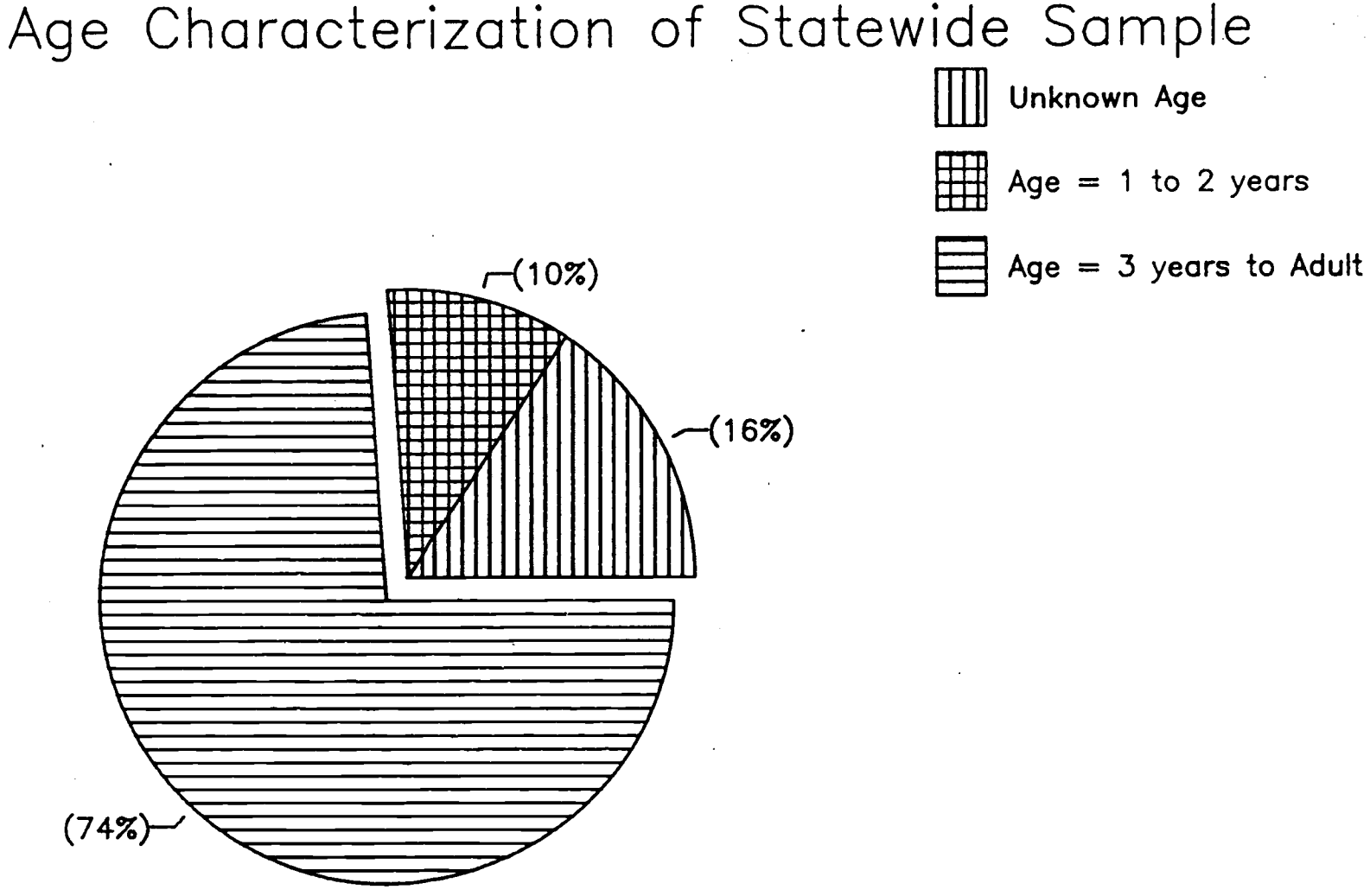

Figure 1. Age characterization of the Kansas cattle survey sample.

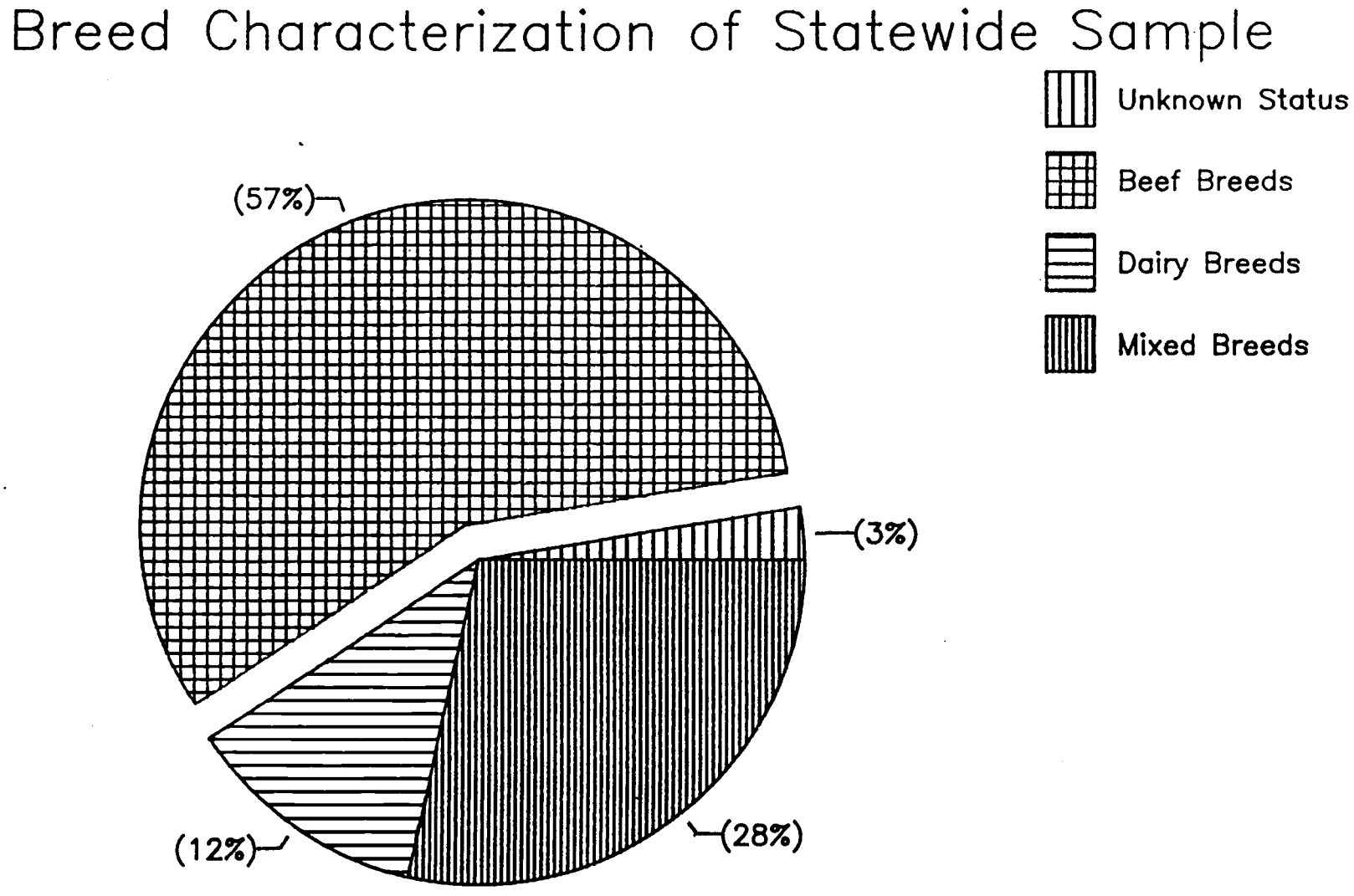

Figure 2. Breed characterization of the Kansas cattle survey sample.

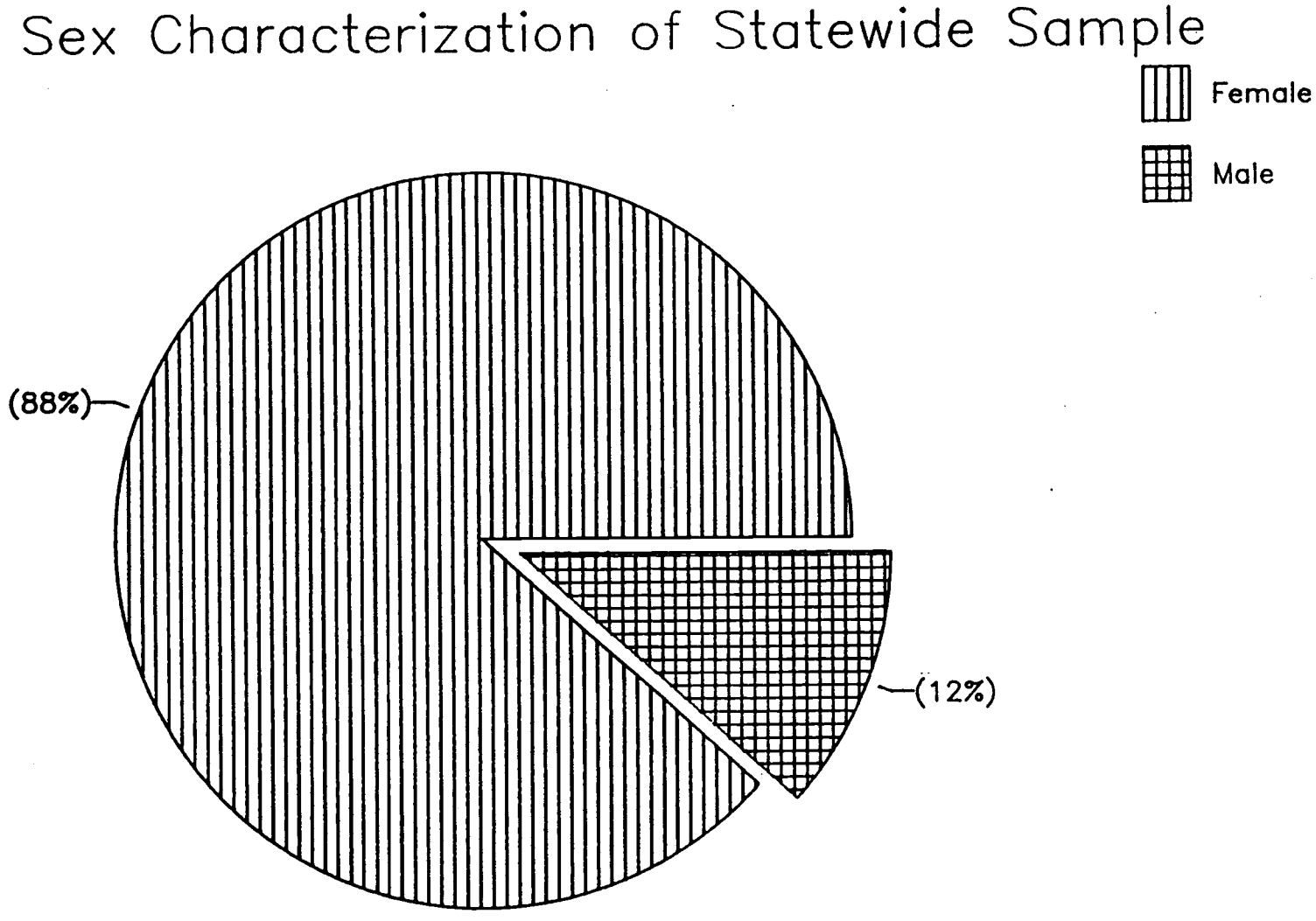

Figure 3. Sex characterization of the Kansas cattle survey sample.

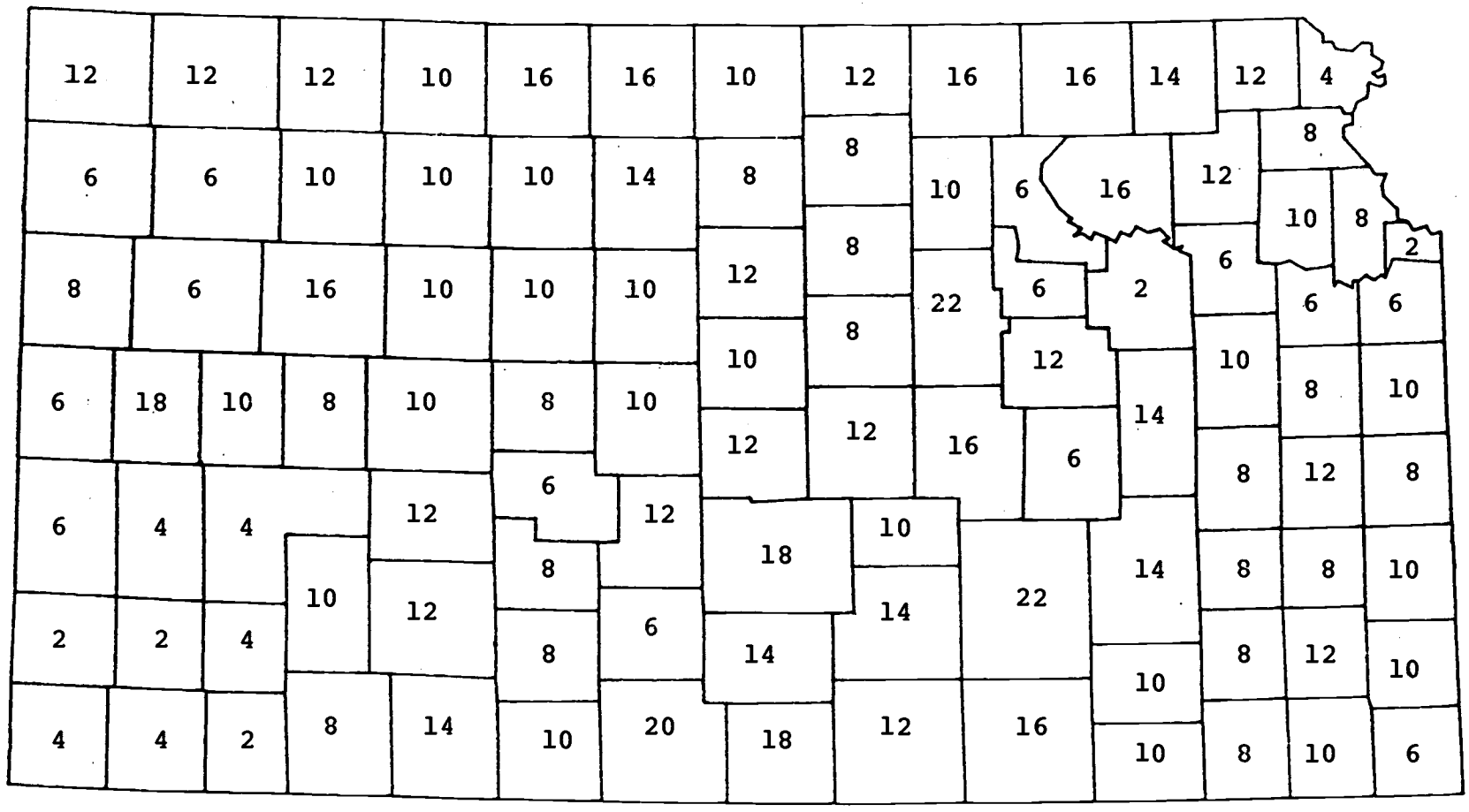

Figure 4. Number of cattle selected from each county for the Kansas survey sample.

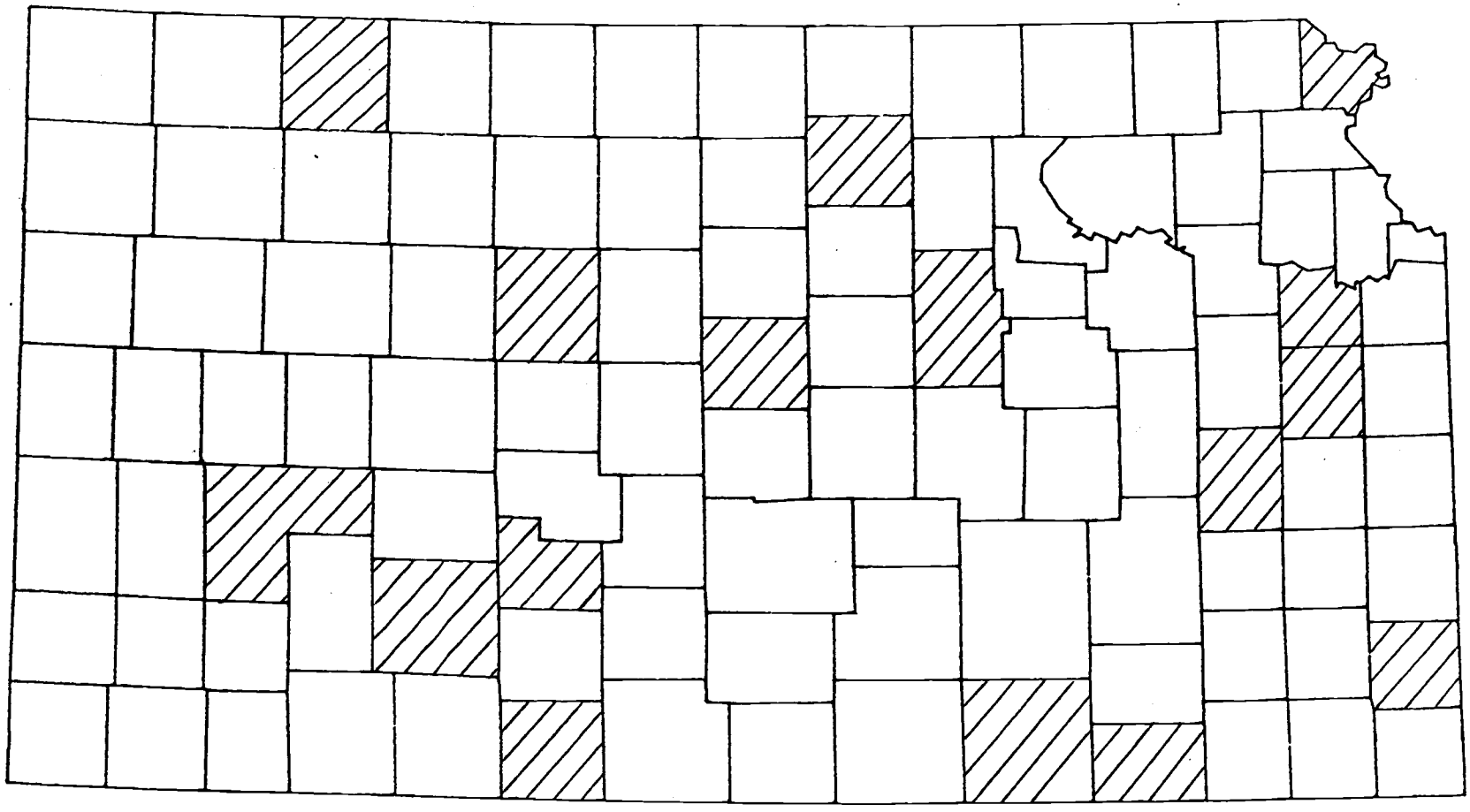

Figure 5. Kansas counties represented in the subsample.

was heat inactivated at 56°C for 30 minutes prior to utilization. The inoculated cells were incubated in capped flasks at 37°C for 12 to 48 hours until viral CPE was observed on 80-90% of the Vero cell monolayer. At this point, the infected cells were frozen to -70°C and then thawed to room temperature. The resultant mixture of virus and cells was clarified by centrifugation at 800G for 10 minutes. The supernatant portion was mixed, placed in sterile vials, and stored at -70°C.

Virus titration

Serial 1:10 dilutions of a working virus stock were made in MEM with 25ul of each dilution being placed in 8 wells of a 96-well tissue culture plate (Corning). Each well containing a virus dilution also received 25ul of MEM and 100ul of a 200,000 to 400,000 cells/ml suspension of Vero cells in MEM containing 3% FBS. Cell control wells received 50ul of MEM, no virus, and 100ul of the cell suspension Figure 6 details the plate layout for this procedure.

Plates were incubated at 37°C in a 95% air and 5% CO_2 moisture chamber for 72 hours. Any amount of CPE observed in a well after incubation indicated positive virus infection. For a valid test, cell control wells had to remain free of CPE. Virus titer was based upon the tissue culture infective dose capable of producing CPE in 50% of the inoculated wells ($TCID_{50}$). The Spearman-Karber method for calculating virus titer was employed and the formula is detailed as follows (29):

$$\text{Log 50\% EP} = \text{Log of highest dilution showing CPE in 100\% of wells} - \left(\frac{\text{Sum \% CPE at each dilution starting with last 100\% response}}{100} - .5\right)(\text{Log of dilution factor})$$

0	-1	-2	-3	-4	-5	-6	-7	-8	-9	-10	CC
0	-1	-2	-3	-4	-5	-6	-7	-8	-9	-10	CC
0	-1	-2	-3	-4	-5	-6	-7	-8	-9	-10	CC
0	-1	-2	-3	-4	-5	-6	-7	-8	-9	-10	CC
0	-1	-2	-3	-4	-5	-6	-7	-8	-9	-10	CC
0	-1	-2	-3	-4	-5	-6	-7	-8	-9	-10	CC
0	-1	-2	-3	-4	-5	-6	-7	-8	-9	-10	CC
0	-1	-2	-3	-4	-5	-6	-7	-8	-9	-10	CC

Figure 6. Microtiter plate layout for the virus titration procedure.

Representation of a 96-well tissue culture plate utilized for titration of virus stocks. The number representing each well is the log of the virus dilution placed in that well. Each virus dilution is placed in replicates of eight wells with the last column of the plate being utilized for cell controls (CC).

For example, if 7 of 8 wells demonstrated CPE at the 10^{-6} virus dilution and 100% and 0% CPE responses were observed at 10^{-5} and 10^{-7} dilutions respectively, the titer would be calculated as follows:

$$\text{Log 50\% EP} = \text{Log } 10^{-5} - \left(\frac{100 + 87.5 + 0}{100} - .5\right) (\text{Log } 10)$$

$$\text{Log 50\% EP} = -6.375$$

This value indicates that the virus dose was $1 \times 10^{6.375}$ or 2.37×10^{6} $TCID_{50}$/.025ml of working stock. The magnitude of titer in this example is typical of those produced by the serotypes used in this study.

Cross-neutralization test

Antigenic identity and specificity of the working virus stocks were determined by cross-neutralization testing. Antiserum to the SMSV-1, 2, 4-13, Tillamook, VESV A-D and VESV F-K types was tested against the prepared virus stocks. All hyperimmune sera were produced by the Plum Island Foreign Animal Disease Diagnostic Laboratory, Greenport, NY except for the Tillamook and SMSV-13 types which were generated at the Central Laboratory for Calicivirus Studies, Oregon State University, College of Veterinary Medicine, Corvallis, OR.

For this test, all heterologous antiserum (hyperimmune sera produced against serotypes other that the virus stocks) was heat inactivated and diluted 1:5 in MEM. Similar dilutions were made of the homologous antisera (hyperimmune sera produced against the virus stocks) with additional serial 1:2 dilutions in MEM containing 20% FBS carried out so that 50% EP titers could be determined. These titers were used as the basis for making 20 antibody unit dilutions of the homologous antisera for later use as positive controls. The approximate titers of

all antisera ranged from 1:200 to 1:5600 as previously determined by the Plum Island and Calicivirus Research Laboratories.

The neutralization procedure used was an adaptation of a general protocol developed by House (51). In this procedure, a 25ul volume of each heterologous antiserum dilution was placed in four separate wells of a 96 well plate. To three of the four wells, 25ul of challenge virus (100 $TCID_{50}$/.025ml) was added with the fourth well receiving 25ul of MEM and serving as a toxic serum control. A Fetal bovine serum control was employed for each test with four wells receiving 25ul of a 1:5 dilution of FBS. Three of these wells also received 25ul of challenge virus and the fourth well received 25ul of MEM thus serving as a toxic serum control. Homologous antisera dilutions were applied to the plate in the same manner as the heterologous antisera except that the toxic serum control well was omitted for each 1:2 serial dilution. Cell controls were prepared in the same manner as in the virus titration procedure.

To determine the effective virus challenge dose of the test, a virus control (back-titration) procedure was performed. For the back-titration, the virus challenge dose was serially diluted 1:10 to 10^{-3} in MEM. Each dilution was placed in replicate wells containing 25ul of MEM. The 10^{-2} dilution of virus represents the 1 $TCID_{50}$ dose level. In order to obtain a more accurate assessment of the 50% EP, 32 wells received this virus dilution. The layout of a 96-well plate used for cross-neutralization testing is shown in Figure 7.

After application of virus, the plates were gently agitated and incubated at 37°C for one hour in a 95% air and 5% CO_2 moisture chamber. Cells were applied at the end of the incubation period with all subsequent procedures being the same as those used for virus titrations.

HO	HE	HE	HE	HE	HE	0	-2	-2	-2	-2	-3
HO	HE	HE	HE	HE	HE	0	-2	-2	-2	-2	-3
HO	HE	HE	HE	HE	HE	0	-2	-2	-2	-2	-3
SC	SC	SC	SC	SC	SC	0	-2	-2	-2	-2	-3
80	160	320	640	FBS	CC	-1	-2	-2	-2	-2	-3
80	160	320	640	FBS	CC	-1	-2	-2	-2	-2	-3
80	160	320	640	FBS	CC	-1	-2	-2	-2	-2	-3
80	160	320	640	SC	CC	-1	-2	-2	-2	-2	-3

Figure 7. Microtiter plate layout for the cross-neutralization test.

Representation of a 96-well tissue culture plate utilized for virus cross-neutralization testing. Wells receiving homologous antiserum are represented by HO and HE designates those wells receiving heterologous antiserum. The HO and HE antisera are placed in replicates of four wells with each HE replicate representing antiserum to a specific serotype. The two and three digit numbers represent reciprocal values for 1:2 serial dilutions of HO antiserum. In many cases, higher dilutions were needed to determine the HO antibody 50% EP. Fetal bovine serum control wells are represented by FBS, SC designates toxic serum control wells, and cell control wells are represented by CC. Note that SC wells are not included for HO antiserum titration. Single digit numbers represent the virus back-titration and each number is the log of the challenge virus dilution placed in that well. Note that 32 wells are used for the 10^{-2} dilution of challenge virus.

For a test to be valid, the following requirements had to be met. First, the cell and toxic serum control wells had to be free of CPE with the FBS controls showing evidence of viral CPE. In addition, the effective virus challenge dose had to fall within the range of 100-450 $TCID_{50}$ as indicated by the back-titration. The virus challenge dose was calculated by the same Spearman-Karber formula used for virus stock titer determination. For example, if 24 of 32 wells demonstrated CPE at the 10^{-2} virus dilution level and 100% and 0% CPE were observed at the 10^{-1} and 10^{-3} dilutions of virus respectively, the titer would be as follows:

$$\text{Log } 50\% \text{ EP} = \text{Log } 10^{-1} - \left(\frac{100 + 75 + 0}{100} - .5\right) (\text{Log } 10)$$

$$\text{Log } 50\% \text{ EP} = -2.25$$

This value indicates that the effective virus titer for the test was $1 \times 10^{2.25}$ or 178 $TCID_{50}$.

A tabular representation of the cross-neutralization results was carried out. The 50% EP titer for the homologous antisera was calculated by the same method as used for titering Kansas screen test positive sera. Although the SMSV-14 virus stock was not tested, its characterization as a distinct serotype was demonstrated by previous cross-neutralization testing. Earlier work also characterized the titer of the furnished SMSV-14 antiserum so that an appropriate dilution could be made and used as a positive control (83).

Neutralization screening test

Cattle sera were diluted 1:5 in MEM and heat inactivated at 56°C for 30 minutes. Each test serum was applied to a 96-well plate in the

same way as heterologous antiserum in the cross-neutralization procedure. The effective serum dilution for the screening test was 1:10 as an equal volume of challenge virus placed in each well diluted the serum by a additional factor of 1:2. The toxic serum, cell, and FBS controls were utilized in the same way as in the cross-neutralization procedure. Additional positive and negative serum controls were included in the test and were applied to the plate in the same manner as the test sera. Homologous antisera, diluted to 20 antibody units in MEM containing 20% FBS, were used as the positive control. Known negative serum obtained from a calf and diluted 1:5 in MEM was utilized as the negative control. Figure 8 illustrates the plate layout for this test. All other procedures and test requirements were identical to those of the cross-neutralization test. For a valid test, the positive control serum should protect cells from CPE while the negative control should allow viral infection.

Results were read to both 67% and 100% antibody EP's due to the large differences in prevalence rates observed at these two EP's. Fifty percent EP's could not be described at the 1:10 serum dilution level because only three wells were utilized for each test serum in this assay. Therefore, any sample that provided protection against CPE in 2 of the 3 wells was considered to be positive for antibody to a 67% EP and protection in all three wells indicated that the serum was positive to a 100% EP. Confidence intervals at the 95% level were constructed for each antibody prevalence using the following formula for the construction of population proportion (p) confidence intervals (33):

$$\text{Prevalence} +/- (1.96)\sqrt{\frac{p(1-p)}{\text{number in sample } (n)}}$$

TS	TS	TS	TS	TS	TS	0	-2	-2	-2	-2	-3
TS	TS	TS	TS	TS	TS	0	-2	-2	-2	-2	-3
TS	TS	TS	TS	TS	TS	0	-2	-2	-2	-2	-3
SC	SC	SC	SC	SC	SC	0	-2	-2	-2	-2	-3
TS	TS	+C	-C	FBS	CC	-1	-2	-2	-2	-2	-3
TS	TS	+C	-C	FBS	CC	-1	-2	-2	-2	-2	-3
TS	TS	+C	-C	FBS	CC	-1	-2	-2	-2	-2	-3
SC	SC	SC	SC	SC	CC	-1	-2	-2	-2	-2	-3

Figure 8. Microtiter plate layout for neutralization screening test.

Representation of a 96-well tissue culture plate utilized for the neutralization screening test. Wells designated TS received a 1:5 dilution of test serum with each serum being placed in replicates of four wells. Positive control wells receiving 20 antibody units of homologous antiserum are labeled +C and negative control wells are labeled -C. Fetal bovine serum control wells are represented by FBS, SC designates toxic serum controls, and cell controls are represented by CC. Each single digit number represents the virus back titration and each number is the log of the challenge virus dilution placed in that well. Note that 32 wells are used for the 10^{-2} dilution of challenge virus.

Graphical and tabular representations of antibody prevalence, multiple serotype exposures, and the statewide distribution of exposure were carried out. Since it was found that the virus challenge $TCID_{50}$ significantly affected the number of screen test positive sera detected, a graphical representation of this effect on 164 sera was carried out.

Neutralization titering test

Antibody titrations were performed on sera screen test positive to SMSV-5, 7, 14 and the Tillamook serotype at a 100% EP. These sera were further serially diluted 1:2 in MEM containing 20% FBS. The test procedures and requirements were the same as those of the screening test except that toxic serum controls were not utilized if the serum samples were shown to be non-toxic on the screening test. Figure 9 illustrates the plate layout for this test. Titers were reported at a 50% EP using the Spearman-Karber method for 50% EP antibody titer determination (51). The formula used is as follows:

$$\text{Log 50\% EP} = \text{Log of highest dilution with complete protection} + \left(\frac{\text{Sum \% protection including last dilution with 100\% protection}}{100} - .5\right)(\text{Log of dilution factor})$$

For example, if 3 of 4 wells were protected from CPE by serum at a 1:40 dilution level and the 1:20 and 1:80 serum dilutions gave 100% and 0% protection respectively, then the titer would be calculated as follows.

$$\text{Log 50\% EP} = \text{Log } 20 + \left(\frac{100 + 75 + 0}{100} - .5\right)(\text{Log } 2)$$

$$\text{Log 50\% EP} = 1.676$$

This value indicates that the 50% EP antibody titer was 1:47. Graphical and tabular representations of 50% EP titers were carried out.

20	40	80	160	320	640	0	-2	-2	-2	-2	-3
20	40	80	160	320	640	0	-2	-2	-2	-2	-3
20	40	80	160	320	640	0	-2	-2	-2	-2	-3
20	40	80	160	320	640	0	-2	-2	-2	-2	-3
+C	-C	FBS	CC	CC	CC	-1	-2	-2	-2	-2	-3
+C	-C	FBS	CC	CC	CC	-1	-2	-2	-2	-2	-3
+C	-C	FBS	CC	CC	CC	-1	-2	-2	-2	-2	-3
SC	SC	SC	CC	CC	CC	-1	-2	-2	-2	-2	-3

Figure 9. Microtiter plate layout for titration of neutralizing antibody.

Representation of a 96-well tissue culture plate utilized for titering neutralizing antibody found in screen test positive sera. Serial 1:2 dilutions of test sera were placed in replicates of four wells with each two and three digit number representing the reciprocal of the serum dilution used. Positive control wells receiving 20 antibody units of homologous antiserum are labeled +C and negative control wells are labeled -C. Fetal bovine serum control wells are represented by FBS, SC designates toxic serum controls, and cell control wells are represented by CC. Single digit numbers represent the virus back-titration and each number is the log of the challenge virus dilution placed in that well. Note that 32 wells are used for the 10^{-2} dilution of challenge virus.

RESULTS

Cross-neutralization testing revealed that each virus stock used in this study was of correct identity and not contaminated with other calicivirus serotypes. Using this test system, antigenic cross-reactivity was not observed when testing each serotype against high titer heterologous antisera. Homologous antisera titers were found to range from 1:160 to 1:5120 at 50% antibody EP (Table 6).

Screen test results revealed that many test sera possessed neutralizing antibody to several marine calicivirus serotypes. The number of sera positive to each serotype was variable with the largest proportions of positives found to have antibody against SMSV-5, 8, and 13. The antibody EP at which the screen test was interpreted significantly affected the number of positive sera reported (Figures 10 and 11).

Based on 100% antibody EP screen test results for both the statewide sample and subsample, 95% confidence intervals for the prevalence of exposure in Kansas cattle to each serotype were calculated. The confidence intervals were; 7.3%-10.7% for SMSV-5, 0.3%-1.5% for SMSV-7, 24.1%-38.5% for SMSV-8, 0.8%-6.8% for SMSV-13, .02%-0.8% for SMSV-14 and >0%-0.6% for TCV. Confidence intervals were larger for exposure to the SMSV-8 and SMSV-13 serotypes as prevalence values were based only on subsample results. Although no antibody to SMSV-1 was detected in subsample sera, the lack of activity of this serotype in Kansas cattle cannot be assumed as the subsample may not have been large enough to detect its presence.

Table 6. Cross-neutralization Test Results

Antiserum Type	Serotype SMSV-1	SMSV-5	SMSV-7	SMSV-8	SMSV-13	TCV
SMSV-1	320*	-	-	-	-	-
SMSV-2	-	-	-	-	-	-
SMSV-4	-	-	-	-	-	-
SMSV-5	-	320*	-	-	-	-
SMSV-6	-	-	-	-	-	-
SMSV-7	-	-	640*	-	-	-
SMSV-8	-	-	-	5120*	-	-
SMSV-9	-	-	-	-	-	-
SMSV-10	-	-	-	-	-	-
SMSV-11	-	-	-	-	-	-
SMSV-12	-	-	-	-	-	-
SMSV-13	-	-	-	-	160*	-
Tillamook	-	-	-	-	-	5120*
VESV-A	-	-	-	-	-	-
VESV-B	-	-	-	-	-	-
VESV-C	-	-	-	-	-	-
VESV-D	-	-	-	-	-	-
VESV-F	-	-	-	-	-	-
VESV-G	-	-	-	-	-	-
VESV-H	-	-	-	-	-	-
VESV-I	-	-	-	-	-	-
VESV-J	-	-	-	-	-	-
VESV-K	-	-	-	-	-	-

*Reciprocal of the antiserum dilution at which 50% of the inoculated wells were protected from viral CPE.
(-) Represents failure of the antiserum to protect cells from viral CPE.

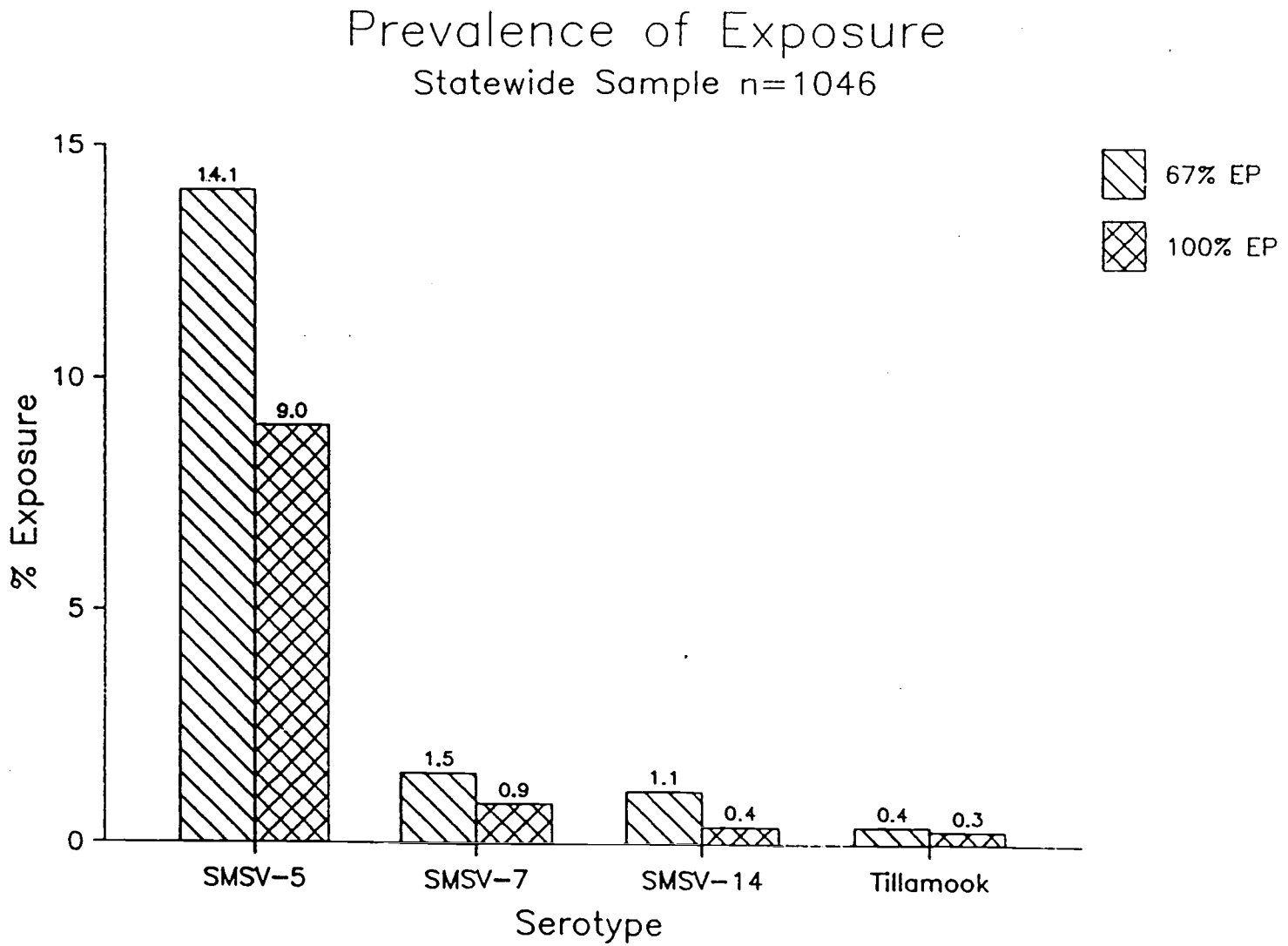

Figure 10. 67% and]00% end point antibody prevalences to three types of San Miguel sea lion virus and the Tillamook serotype in the statewide Kansas cattle sample.

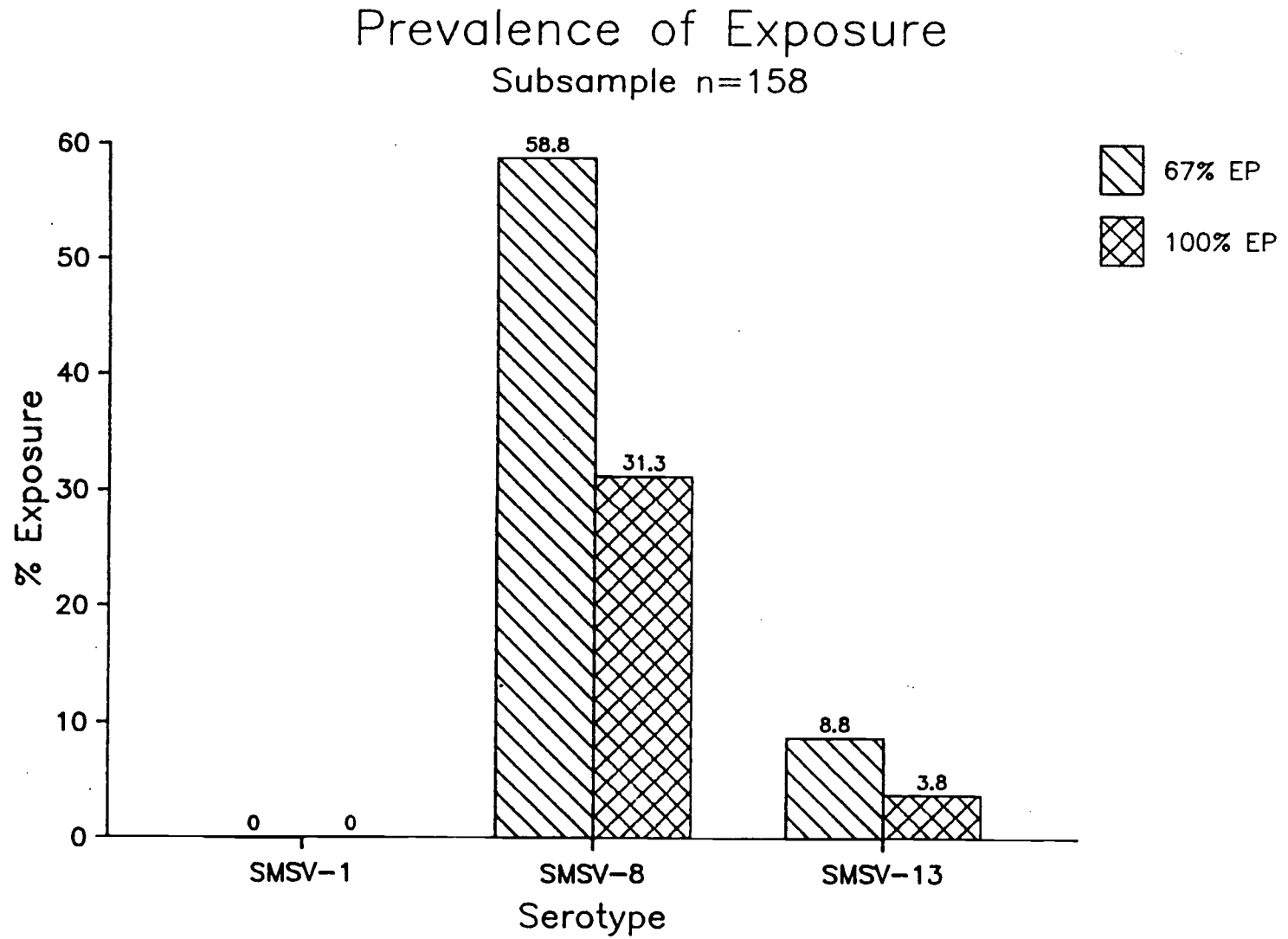

Figure 11. 67% and 100% end point antibody prevalences to three types of San Miguel sea lion virus in the Kansas cattle subsample.

Confidence intervals for combined exposure of cattle to the SMSV-5, 7, 14 and Tillamook serotypes on the basis of age, breed and sex characteristics have been calculated and are given in Table 7. The exposure prevalence rates are based on 67% antibody EP screen test results for the statewide sample. Non-overlapping confidence intervals were found for only one category suggesting that animals 1 to 2 years of age appear to have lower levels of combined exposure than older animals. Since the number of sera representing each category was small and no attempt was made to obtain a representative sample of Kansas cattle on the basis of these characteristics, exposure relationships of this type may not be accurate.

Several individual screen test positive sera of the subsample possessed 67% EP neutralizing antibody to multiple marine calicivirus serotypes (Figure 12). It was found that a few sera possessed antibody to as many as three serotypes. When titered, these sera usually had low levels of antibody to each serotype. One serum, however, demonstrated a very high 50% EP titer of 1:1280 to SMSV-7, a titer of 1:14 to SMSV-5, and had detectable antibody to either SMSV-14 or TCV. From Figure 12, it is interesting to note that approximately 66% of the subsample sera tested possessed antibody to at least one of the seven serotypes at the 67% antibody EP.

From statewide sample screen test results, the distribution of SMSV-5 exposures in Kansas cattle appears to be widespread. The degree of distribution was found to be dependent upon the antibody EP interpretation used. Cross-hatching in Figures 13 and 14 designates counties represented by cattle sera possessing antibody to SMSV-5 at the 67% and 100% EP's respectively. The widespread character of SMSV-5

Table 7. Antibody Prevalence Values for Age, Breed, and Sex Characteristics Represented in the Statewide Sample.

AGE

Category	Positive Samples*	Total Samples	Antibody Prevalence	95% Conf. Interval
1-2 Years	8	109	7.3%	2.4%-12.2%
3 Years-Adult	132	774	17.1%	14.4%-19.8%
Unknown Status	30	163	18.4%	6.6%-24.3%
Total	170	1046	16.3%	14.1%-18.5%

BREED

Category	Positive Samples*	Total Samples	Antibody Prevalence	95% Conf. Interval
Dairy	13	127	10.2%	4.9%-15.5%
Beef	110	595	18.5%	15.4%-21.6%
Mixed	44	297	14.8%	10.8%-18.8%
Unknown Status	3	27	11.1%	>0%-22.9%
Total	170	1046	16.3%	14.1%-18.5%

SEX

Category	Positive Samples*	Total Samples	Antibody Prevalence	95% Conf. Interval
Male	13	124	10.5%	5.1%-15.9%
Female	157	922	17.0%	14.6%-19.4%
Total	170	1046	16.3%	14.1%-18.5%

*All positive samples were screen test positive at a 67% end point to either SMSV-5, 7, 14 or the Tillamook serotype.

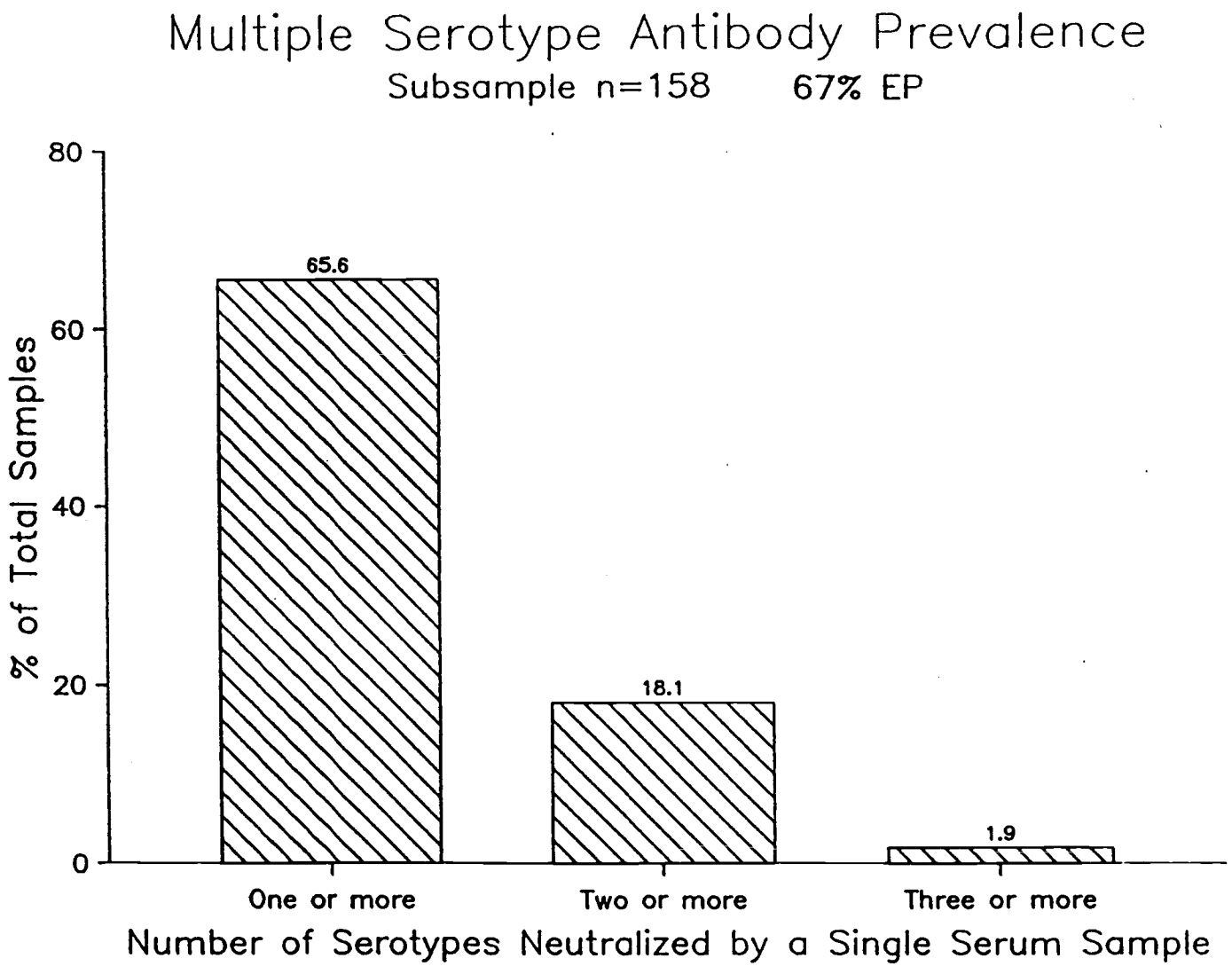

Figure 12. Frequency of multiple serotype exposure to six types of San Miguel sea lion virus and the Tillamook serotype in the Kansas cattle subsample.

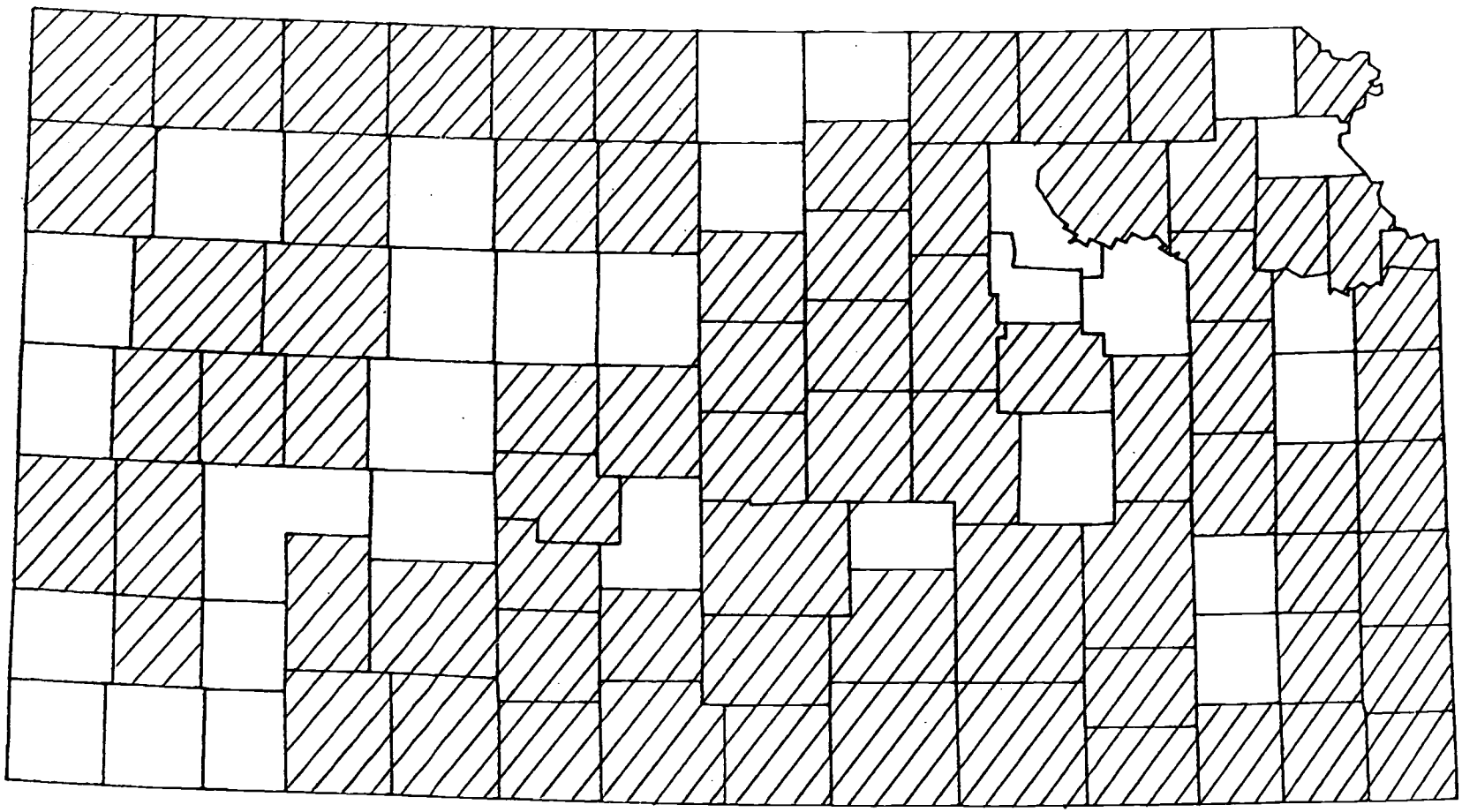

Figure 13. Distribution of Kansas counties with cattle demonstrating antibody to San Miguel sea lion virus type 5 at a 1:10 serum dilution read to a 67% end point.

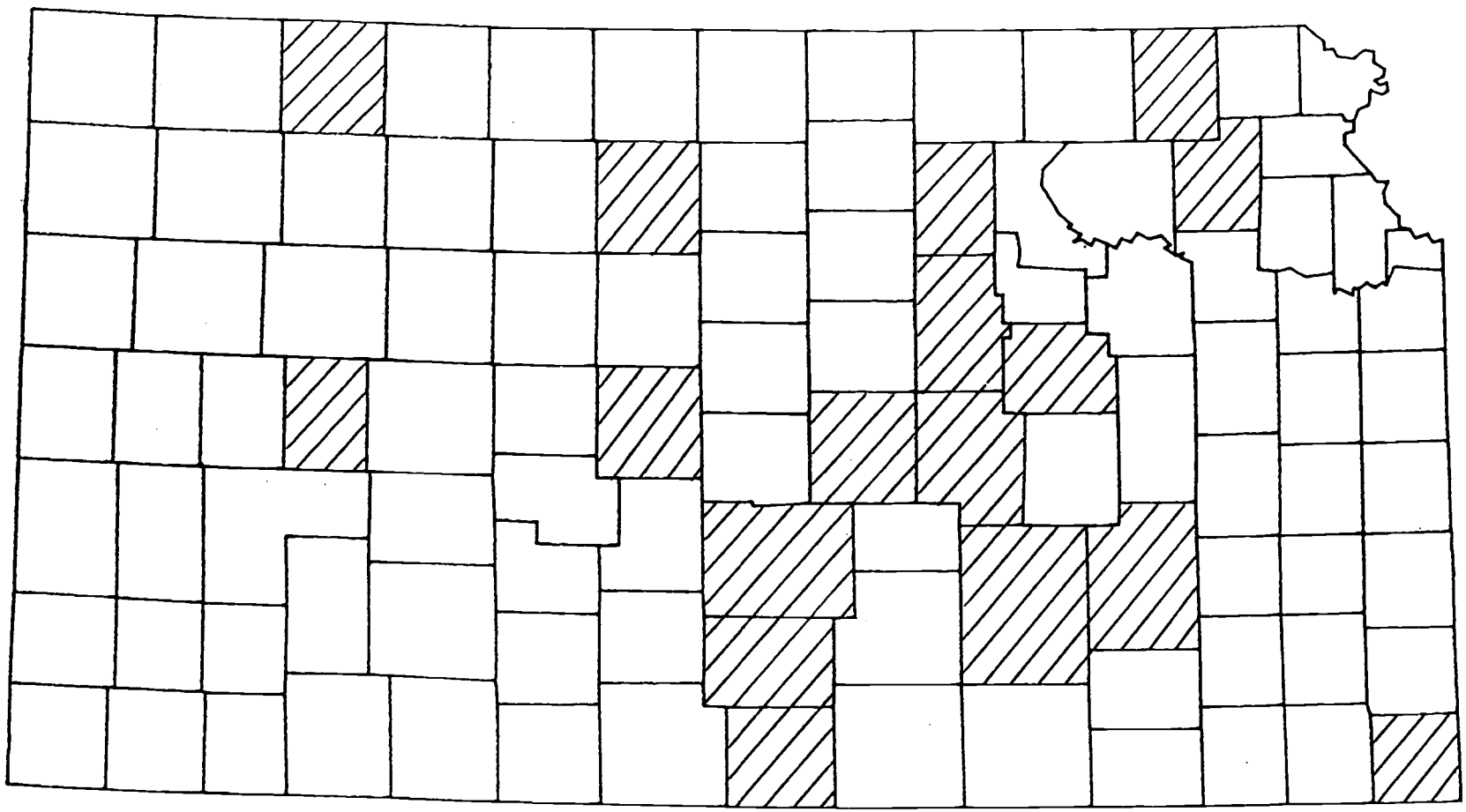

Figure 14. Distribution of Kansas counties with cattle found to have antibody to San Miguel sea lion virus type 5 using a 1:10 serum dilution read to a 100% end point.

exposures in Kansas cattle may serve as an indication of the distribution of exposure to other marine caliciviruses. The apparent lack of SMSV-5 exposure in some counties may not mean that this agent is not present, considering the limited number of samples selected from each county. An analysis for trends in exposure within the state was carried out based on comparing the total number of screen test positive animals to the total number of stock cattle in each county. Exposure trends were not readily apparent; however, the limited number of samples taken from each county makes such an analysis potentially very inaccurate.

The effect of the challenge virus $TCID_{50}$ on the number of screen test positive sera detected was significant within a narrow TCID range. Using 164 serum samples, a three fold difference in the number of SMSV-5 100% EP screen test positive sera was observed using a 75 $TCID_{50}$ to 421 $TCID_{50}$ challenge virus range (Figure 15). This dose effect can greatly affect the repeatability of the serum neutralization test. In this study, it was observed that the effective virus dose was very sensitive to slight changes in test conditions and procedure.

Serum samples of the statewide sample found screen test positive at the 100% EP antibody level to SMSV-5, 7, 14, and the Tillamook serotype were titered to a 50% EP. Resultant titers were variable ranging from 1:14 to 1:1280 with the majority found to be in the 1:10 to 1:20 range (Figure 16 and 17). The high titer found to SMSV-7 could be the result of recent infection. Table 8 lists the number of samples falling into specific titer ranges for each serotype. From this table it is apparent that few sera were titered and that the calculation of average titers illustrated in Figures 16 and 17 are misleading, especially with respect

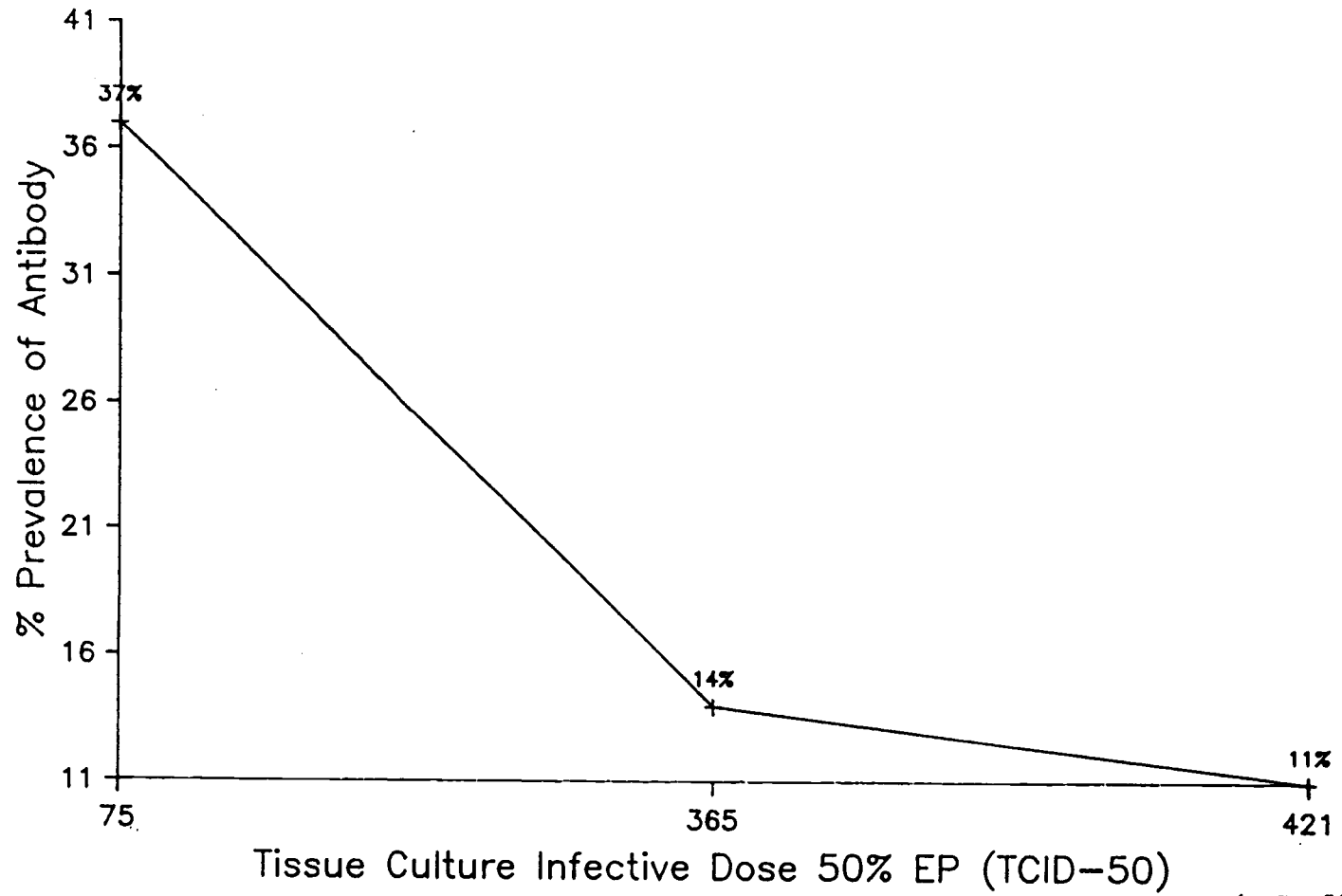

Figure 15. Effect of the 50% end point tissue culture infective dose of challenge virus (TCID-50) on the number of screen test positive serum samples detected.

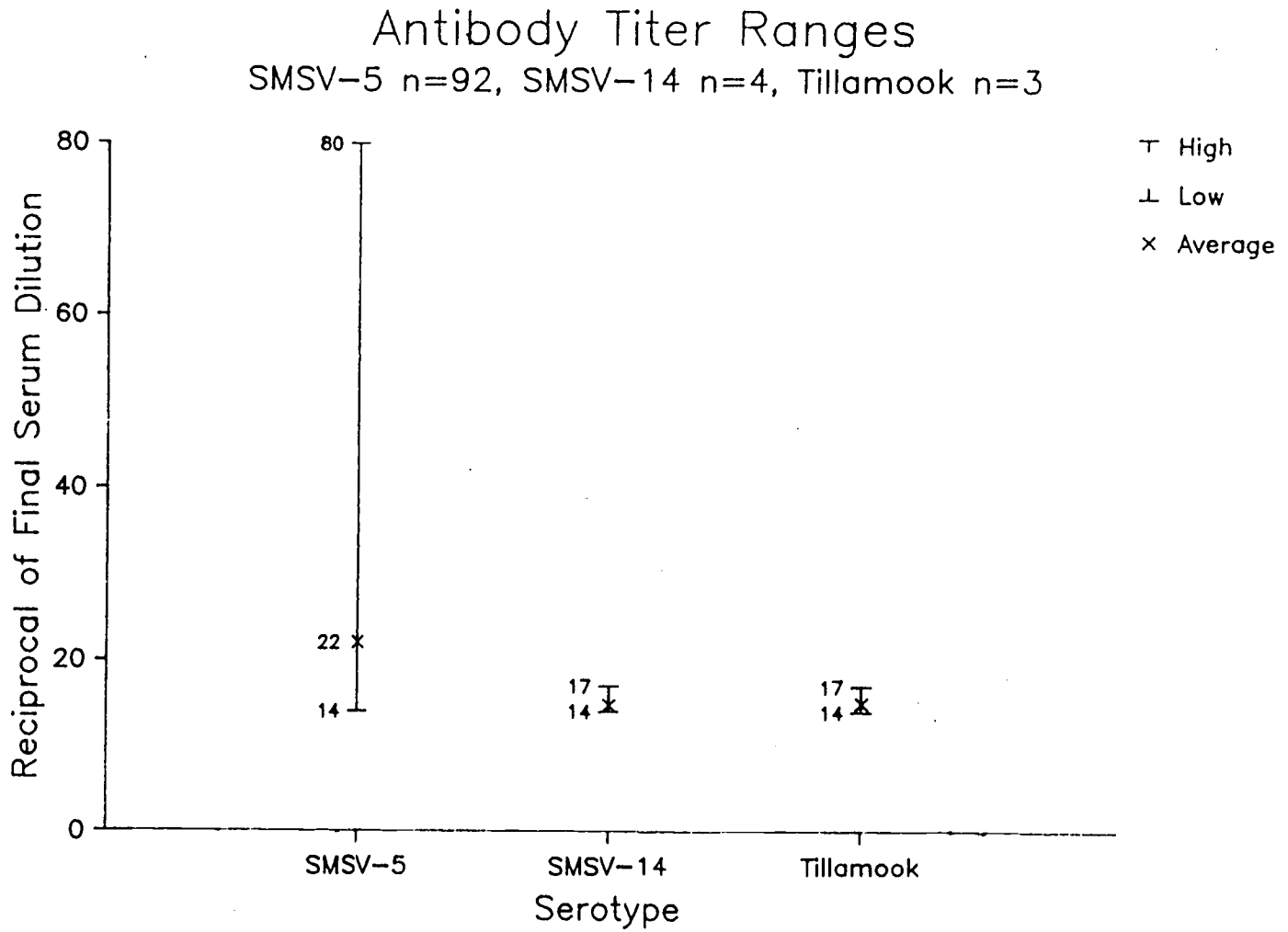

Figure 16. Neutralization antibody 50% end point titer ranges to San Miguel sea lion virus types 4 and 5 and the Tillamook serotype for the statewide Kansas cattle sample.

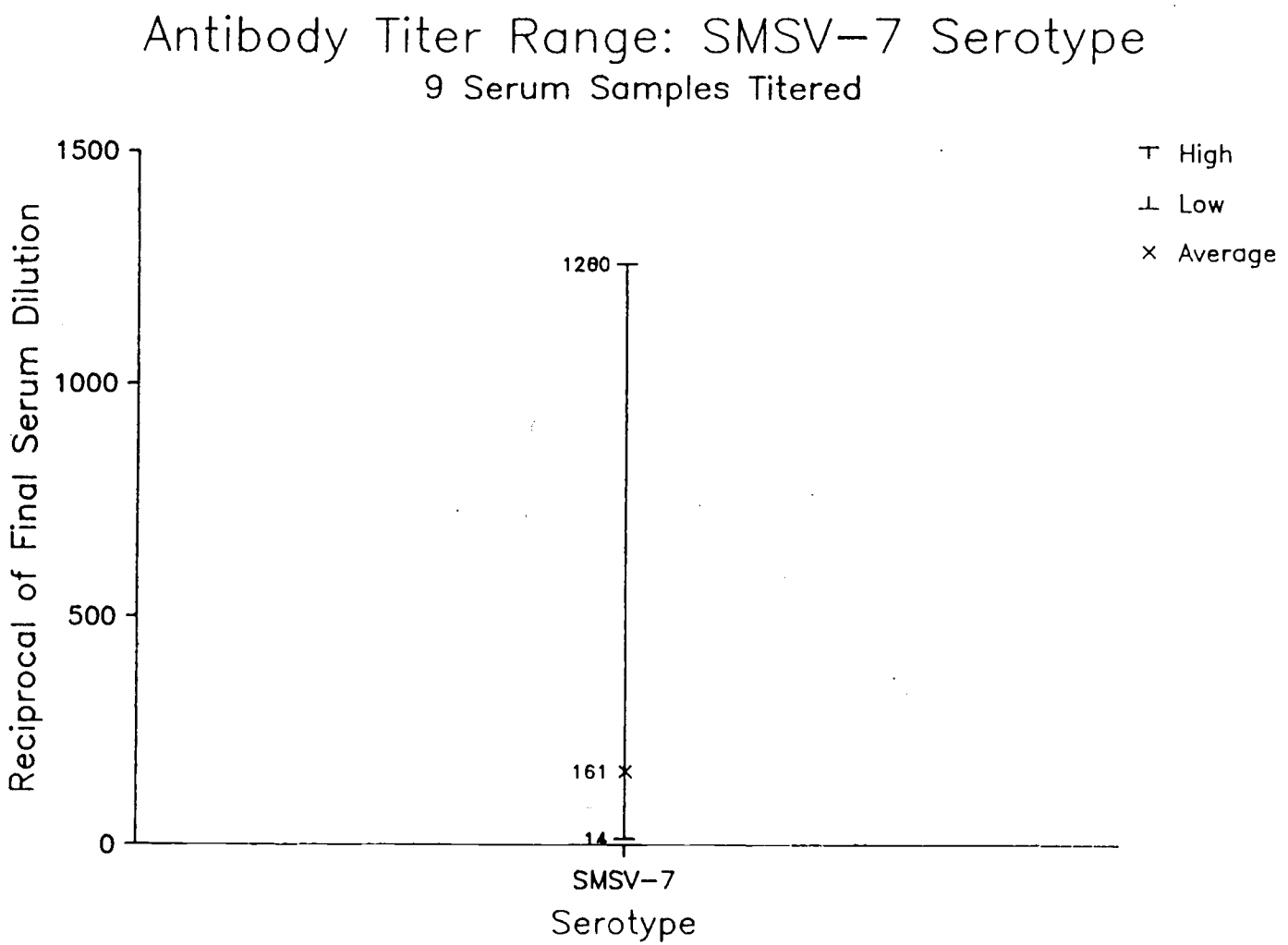

Figure 17. Neutralization antibody 50% end point titer range to San Miguel sea lion virus type 7 for the statewide Kansas cattle sample.

Table 8. 50% End Point Titers of Statewide Sample Screen Test Positive Sera to Four Calicivirus Serotypes

Serum Dilution*	SMSV-5	SMSV-7	SMSV-14	Tillamook
1:10	39	3	3	2
1:20	45	5	1	1
1:40	7	0	0	0
1:80	3	0	0	0
1:160	0	0	0	0
1:320	0	0	0	0
1:640	0	0	0	0
1:1280	0	1	0	0
Total**	94	9	4	3

*Observed values were adjusted to fit into the nearest titer category.
**Only 100% antibody EP screen test positive sera were titered.

to the SMSV-7 serotype. Since many sera had titers to SMSV-5, average titers to this serotype may be a better indicator of typical titers in cattle.

DISCUSSION

The serum neutralization antibody assay was found to have several limitations when applied to this study. For instance, the exposure prevalences reported are not absolute values, as it was clearly demonstrated that a change in antibody EP interpretation of the test significantly affected the number of test positive sera detected. This EP effect was probably due to the typically low levels antibody present in the sera. Accepted exposure prevalence values were based on the number of sera found positive for antibody at a 1:10 dilution using a 100% antibody EP. The 67% and 50% EP's were frequently used in this study to increase the sensitivity of the test so that a more complete characterization of exposure could be carried out.

Serum neutralization test results were also found to be very dependent upon the challenge virus dose used. Again, the magnitude of this effect was probably due to the characteristically low levels of antibody present in the sera. With higher levels of antibody, neutralization test results would be more resistant to changes in virus dosage. In an attempt to determine conservative estimates of exposure, the lowest effective challenge virus dose accepted for any test was 100 $TCID_{50}$.

Serotype specificity of the neutralization test was investigated by using hyperimmune antiserum in the cross-neutralization testing of several virus types. The results confirmed that the neutralization test is serotype specific and that neutralization of calicivirus serotypes by spurious antibody is not likely to occur. If this type of antibody were responsible for the virus neutralizations detected in this study, the

spurious antibody would have to be serotype specific because some sera possessed antibody to several serotypes while others contained antibody to only one type. Since high titer hyperimmune sera did not cross-react with heterologous virus types, it is unlikely that cross-reactivity would occur with the lower levels of antibody found in Kansas cattle sera. Further evidence against the existence of antigenic cross-reactivity in this test system, is the detection of type specific neutralizing titers as high as 1:2560 in naturally infected marine mammals in the absence of antibody to other serotypes (81).

Serum neutralization test results may not be an accurate indicator of in vivo protection. For example, persistent infection in the presence of neutralizing antibody has been shown to occur with these agents (89, 103). However, it was shown in another study that the production of neutralizing antibody correlates with virus clearance (44). Very few studies have investigated the longevity of detectable neutralizing antibody after initial exposure in domestic livestock. It has been reported that neutralizing antibody can persist at high levels for up to 7 months in swine infected with VESV (50). Using this information, cattle found to have neutralizing antibody in this survey may not have experienced recent exposure to these marine caliciviruses.

The prevalence and distribution of calicivirus antibody reported in this study is believed to be a good representation of exposure in Kansas cattle. It is apparent that significant widespread exposure to several serotypes in Kansas cattle is occurring. Although only the distribution of SMSV-5 exposure was characterized, other marine serotypes may also have a widespread presence. From this study, it may be possible to make

some generalizations about the prevalence and distribution of these agents in U.S. cattle. For instance, it is likely that other states, with cattle movements and husbandry practices closely resembling those of Kansas, are experiencing similar exposures to these viruses. Also, if these viruses are entering livestock populations strictly through direct contact from marine sources, it is logical to assume that the extent of exposure in Kansas cattle should be less than that of cattle from states geographically closer to ocean margins.

The implications of detecting antibody to several marine caliciviruses in Kansas cattle are far-reaching. First of all, viral agents known to cause clinical VE in swine on a experimental basis have now been found to be circulating in midwest cattle. The location of the exposed cattle is very near major swine producing areas. Although the disease potential for swine is great, clinical disease has not been reported. Several factors may be responsible for this presumed lack of disease. First of all, swine husbandry methods are quite different now than when VE outbreaks were occurring. Raw-garbage no longer provides a direct avenue for transmission, and confinement operations may effectively isolate swine from other exposed domestic livestock. It is possible that non-pathogenic types are presently circulating in livestock with infection occurring in the absence of visible lesions. If this is the case, then a future switch to more pathogenic types could be devastating to the livestock industry considering the present widespread extent of exposure in cattle to these agents. It is also possible that only low level exposures are occurring thus causing subclinical disease or that vesicular disease in swine is not being reported when it occurs.

Exposure in cattle certainly exists although characteristic vesicular disease has not been reported. Again, this may be due to the activity of non-pathogenic types, low level exposures, or non-reporting of vesicular disease. In addition, these agents may be causing disease syndromes that have undefined etiologies. Without efforts being made to examine diseased animals for evidence of calicivirus infection, this agent's impact on domestic livestock may be overlooked.

Another implication of finding marine calicivirus antibody in cattle is regulatory in nature. From this study, it is apparent that some U.S. cattle have been exposed to a disease agent classified as indistinguishable from a virus that has been eradicated and is considered exotic to livestock. In addition, antibody to the VESV's have been recently detected in several terrestrial species including swine in several western states (Table 5). Serological and virus isolation work also indicates that several serotypes of VESV are circulating in the marine environment. These findings will likely force a re-evaluation of the regulatory classification of these agents.

With the exposure of terrestrial species to marine caliciviruses established, questions quickly arise regarding possible transmission routes and potential disease reservoirs of these agents. To address these questions, it is helpful to understand the extent of marine exposures in relation to land/ocean margins. Knowing the ocean ranges of marine life found to be exposed to caliciviruses (74, 97), the land margins bordering exposed marine environments can be illustrated (Figure 18). From this figure, it is apparent that these land borders are extensive and not confined to the U.S.

Considering the marine environment as a direct source of these

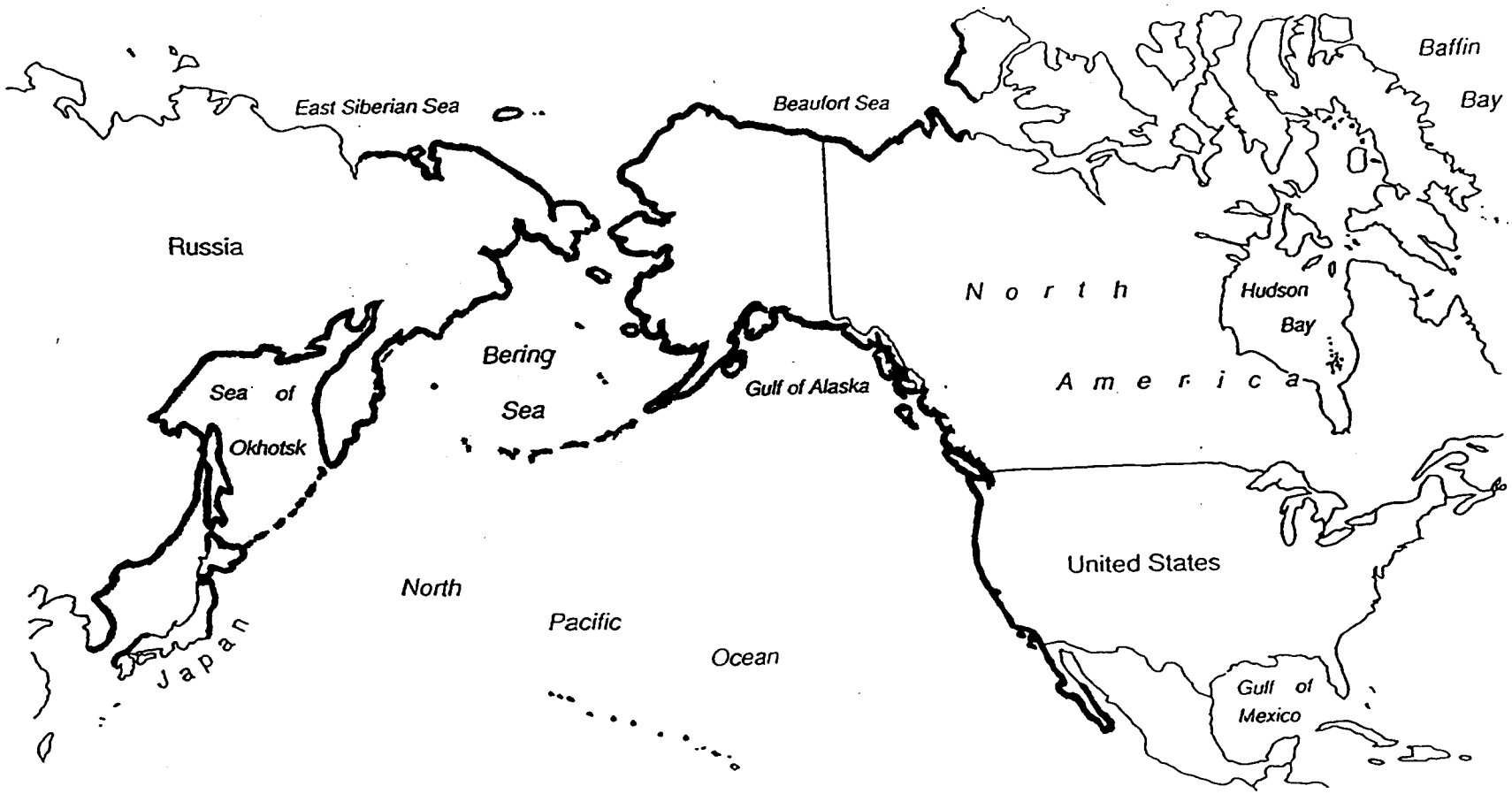

Figure 18. Land margins bordering ocean ranges of known calicivirus exposed marine mammals and fish.

agents, several transmission routes have been suggested. The scavenging of diseased marine carcasses washed up on beaches and the foraging on contaminated material in intertidal surf splash zones by carnivores and herbivores may lead to exposure in these species. Sea water bubbling, which is known to scavenge and concentrate organic particulates, may release these agents as aerosols into prevailing onshore winds. Haul out zones for marine mammals could also provide direct contact opportunities. The movement of feral swine from coastal margins into central California for game hunts has been suggested as an avenue for transmission further inland (78). Inland exposures may also occur from anadromous fish movements, such as those of salmon, and from the distribution of marine fishery by-products (64, 73, 97).

The incorporation of marine by-products into feed supplements is considered the single most important potential route of transmission of marine caliciviruses from ocean sources to domestic livestock (11, 14, 68, 117). For instance, the source of TCV infection in cattle has not been found, however, it is thought that this serotype is of marine origin and that feed supplements played a role its transmission to cattle (14, 17, 74). In addition, it has been suggested that the VESV's are of marine origin and were circulating in marine populations at the time of the VE outbreaks (14). Transmission of VESV to swine from the marine environment may have occurred through the incorporation of infected marine products, such as sea lion carcasses or raw fish scraps, into raw-garbage. Infected fish scraps have been suggested as the most probable cause of the first outbreaks of VE because all affected premises had obtained raw-garbage from port city institutions and restaurants (54, 73, 78).

The search for marine calicivirus reservoirs has been addressed by many studies as it is thought that domestic livestock are aberrant hosts of these agents (14, 74). From these studies, the focus of marine activity appears to be in southern California waters. In addition, the California sea lion has been shown to be the marine mammal species having the most intense involvement with the SMSV's (11, 15, 14, 97). In light of this information, California sea lions were first thought to be a major virus reservoir (97). More recent evidence supports the suggestion that opaleye fish, which serve as food sources to California sea lions, are a primary reservoir for marine caliciviruses (74, 97, 102). Several characteristics of the opaleye fish make it a good candidate reservoir. First of all, the opaleye population is large enough to maintain known calicivirus serotypes on a continuous basis (78, 81). Also, opaleye fish habitat lies almost entirely within the California sea lion range, within the range of the early outbreaks of VE in California, and within the area of greatest observed activity of the SMSV's. In addition, opaleye fish have a close association with California sea lions and have direct access to the terrestrial environment through the distribution of fish scraps generated by the commercial fishing industry (12, 14, 74, 81, 102). It is interesting to note at this point that a very high titer to SMSV-7 was detected in a Kansas cattle serum sample and that this same serotype was also initially isolated from an opaleye fish.

The perceived occurrence of disease in marine populations supports the designation of a California sea lion food source as the reservoir for the SMSV's. For instance, disease outbreaks involving the SMSV's in California sea lions appear to be enzootic as SMSV-5 has been found to

persist in this species for long periods of time (28). In contrast, disease outbreaks involving the SMSV's in northern fur seal populations have been characterized as epizootic (14, 17, 72, 76, 93). Also, different SMSV serotypes have been found to be present in each population at any given time (19, 79, 87, 93, 97). Interestingly, the known food sources for these two species are dissimilar.

The spread of calicivirus to different marine mammal species may be linked by migratory pathways. It has been suggested that the movements of stellar sea lions, northern fur seals, and whales through the southern California active zone may be responsible for the presence of calicivirus serotypes in other areas (15, 97). For example, several disease outbreaks involving specific serotypes have occurred in geographically distant northern fur seal populations following initial appearance in California sea lions (13, 81). It is thought that northern fur seals acquire infection from sharing a sea lion food source, such as the opaleye, while in the calicivirus active zone and then transmit newly acquired virus to other fur seals during and after northward migration to the Pribilof Islands (101). Exposure in other species, such as the walrus, Hawaiian monk seal, and bowhead whale is difficult to explain as these populations have discrete ranges and are essentially isolated from the movements of California sea lions and northern fur seals. In these cases, it has been suggested that the virus is either maintained entirely within the species or that food sources may serve as a virus reservoir (14, 16, 47, 93).

There is speculation that other fish, in addition to the opaleye, may be reservoirs of marine caliciviruses. A preliminary attempt to find calicivirus exposure in 19 species of fish resident to California

shore waters has been carried out. Neutralizing antibody was not found, however, many other species dwelling in California shore waters remain untested (81).

Reservoirs of marine caliciviruses, including the VESV's, appear to have maintained the virus in an antigenically unchanged state for many years. Recent exposure to the same serotypes of VESV found over 40 years ago has been detected in both the marine and terrestrial environments (Tables 3 and 5). Considering this, it seems unlikely that rapid mutation and genetic instability entirely explain the antigenic variability of caliciviruses (12, 14). The SMSV's are no longer considered recent mutants of the VESV's (67), as many SMSV serotypes have been shown to have a long standing presence in both the marine and terrestrial environments (Tables 2, 3 and 5). Using virus isolation as an indicator of high levels of SMSV activity in the marine environment, there is evidence for epidemic cycling of the SMSV's. For example, the same SMSV serotype has been re-isolated from marine mammals 10 years after its initial recovery from the marine environment (72). Pathotype switching of the SMSV's is believed to occur, and has been suggested as an explanation for finding exposure in marine mammal populations to a serotype several years prior to its isolation during severe disease outbreaks (76, 79). Since it is unclear how long neutralizing antibody persists in marine mammals, it is possible that residual antibody from previous outbreaks may explain this detection of prior exposure.

Although studies have revealed that there is supporting evidence for the existence of calicivirus ocean reservoirs and ocean to land transmission, a great deal of uncertainty still exists. In comparison, the transmission of these agents in terrestrial animals and the

definition of possible terrestrial reservoirs have not been well studied. The suggestion that the marine caliciviruses have terrestrial reservoirs and are being transmitted to the marine environment by the natural flow of material to the oceans could certainly be offered. In fact, due to persistent shedding of TCV observed in calves, terrestrial calicivirus reservoirs have already been proposed (17).

Regardless of suspected calicivirus reservoirs, the land/ocean transmission lines appear to be open and operating. The assumption that these agents are confined to the north Pacific Ocean is not realistic as marine mammals do not respect geographical boundaries and are capable of spreading these viruses throughout the world (74, 95). This type of transmission may have occurred when vesicular disease outbreaks in swine, resembling VE, were recently reported in Australia, New Zealand, and Tasmania. It is interesting to note that affected swine had been fed raw or undercooked feedstuffs of marine sources. The causal agent of the disease outbreaks remains undefined (59). It is also unrealistic to assume that all calicivirus serotypes present in the marine and terrestrial environments have been recognized. The total number of serotypes is unknown as new types are continually being isolated. Combining the information from this study, and other work involving marine caliciviruses, several important points emerge. First, extensive exposure to many serotypes, including the VESV's, is occurring in the marine and terrestrial environments. For example, a high level of exposure to SMSV-8 was found in Kansas cattle even though this serotype was first isolated from northern fur seals in Alaska in 1975 and recent exposures have been found in California sea lions, stellar sea lions, a bowhead whale, and walrus. The above example also illustrates that the

host spectrum can be very broad for many of these serotypes, and that transmission between land and ocean appears to be occurring. Another important point is that experimental infection has been shown to produce significant defined disease in several host species. Additionally these agents have demonstrated environmental resistance, antigenic stability, and appear to have had a long standing presence on land and in the sea.

There are many aspects of marine calicivirus activity that are unclear. First, the origins, reservoirs and land-sea transmission routes are not well understood. Also, it is not known how many serotypes of calicivirus exist or what their worldwide distribution is. Finally, all susceptible species and the resultant disease syndromes caused by infection are not known.

Focusing on calicivirus exposures in domestic livestock, several recommendations for further action are apparent. First, the official status of these agents needs to re-evaluated. Since marine caliciviruses exposures, including exposures to some VESV serotypes, have occurred in domestic livestock, these agents can no longer be considered eradicated and foreign to the U.S. Reclassification may affect export markets as this designation will create the appearance that these viruses are unique to U.S. livestock. The widespread exposure of domestic livestock, in the absence of detectable disease, should be further investigated. It is difficult to understand why clinical syndromes are not being detected when characteristic disease in both swine and cattle has been produced by these agents in experimental inoculation studies. To answer this question, better diagnostics for virus detection need to be developed, and an attempt made to define disease syndromes caused by natural infection. This information can

then be used to define the impact of these viruses on the livestock industry. With the risk to the industry known, and epidemiological aspects of the disease well defined, appropriate control measures can be then developed and effectively carried out.

SUMMARY

From the testing of serum samples representing the indigenous cattle population of Kansas, neutralizing antibody to six of seven marine calicivirus serotypes was detected. Significant levels of exposure were found to several serotypes and some sera possessed antibody to multiple serotypes. Antibody titers were found to be relatively low, with most falling in the 1:10 to 1:20 range. The serum neutralization test was shown to be serotype specific with the detection of spurious antibody unlikely. A widespread distribution of exposure to one serotype was found in Kansas cattle and it is possible that other serotypes will have a similar distribution. Based on available information, other U.S. cattle populations having comparable animal movements and husbandry practices would be expected to have similar exposures to these serotypes. Test results were very dependent upon the $TCID_{50}$ of challenge virus. In addition, the antibody end point interpretation significantly altered the values assigned to statewide prevalences and the reported distribution of exposure. Reclassification of the status of the marine caliciviruses should be considered because these agents are not foreign to U.S. Livestock. The widespread exposure of Kansas cattle to these viruses in the absence of reported vesicular disease requires investigation, especially since experimental studies and historical experience indicate that these agents are capable of producing clinical vesicular disease. It is apparent that the impact of these agents on the livestock industry needs to be better defined.

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