The pathogenicity of the canine herpesvirus

by

Howard Thomas Hill

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INTRODUCTION

The pathogenicity of the canine herpesvirus (CHV) has been extensively studied and well documented as it relates to neonatal infections of the dog. There have also been experimental studies with CHV as it affects the adult dog, but the results of these experiments are conflicting. Some of the variation in results may be due to differences in viral isolates. As yet only serological tests have been used to determine the interrelationships between various isolates of CHV. Until more extensive biochemical and biological studies are performed, we must assume that the various CHV isolates are antigenically related as shown by serum-virus-neutralization tests while recognizing that there may be strain differences detectable with other tests.

A report of CHV being isolated from the respiratory tract of dogs with infectious canine tracheobronchitis and a subsequent report of the reproduction of this disease with the same virus (66) stimulated interest in the pathogenicity of this virus in non-neonatal dogs. However, later reports by other workers (2), in which CHV was shown to cause only mild or inapparent respiratory infections, cast the CHV back into its previous role as a significant pathogen of only the neonatal dog.

This study was designed to determine the pathogenicity of CHV-270 in non-neonatal dogs. Particular efforts were

devoted to the pathogenicity of this virus for the genital tract partly because of the implications genital infections of adult dogs have in the transmission of CHV to neonatal dogs. The recent association of a CHV isolated in England from dogs experiencing stillbirths, abortions and infertility (90), was also a stimulus to study the effects of CHV-270 on the genital tract of dogs. The pathogenicity of CHV-270 for the respiratory tract was another aspect of this disease that was studied. Dogs infected with CHV-270 and maintained at normal environmental temperatures were compared with CHV-270 infected dogs maintained at varied environmental temperatures.

In addition to the CHV-270 pathogenicity studies involving the genital and respiratory tracts of non-neonatal dogs, the immunological response to this virus was studied in an attempt to determine the role circulating neutralizing antibodies have on CHV infections of non-neonatal dogs.

LITERATURE REVIEW

History of the Canine Herpesvirus

The canine herpesvirus (CHV) was first isolated by Carmichael, Fabricant and Squire in 1964 from the heart blood, lungs, livers, spleens and kidneys of two litters of pups that were dying of a hemorrhagic disease that the authors thought was caused by a mycoplasma (27). A year later Carmichael, Strandberg, and Barnes reported that certain tissues from these pups that died contained 2 organisms, a virus and a mycoplasma (29). The mycoplasma failed to produce cytopathic effects (CPE) in dog kidney cells (DKC) and it did not kill young pups inoculated orally and intraperitoneally, so they concluded that the mycoplasma was not the etiological agent of this disease. They did, however, determine that the virus, which they identified as a herpesvirus and designated strain F-205, was the etiological agent of the disease. This conclusion was based on the fact that bacteriologically sterile cell-culture fluid from DKC showing viral CPE, when inoculated orally or intraperitoneally, caused the typical pathologic changes in young pups characteristic of the original disease. In 1965 Stewart *et al.* (105) reported the isolation of CHV from dying neonatal dogs and in the same year Spertzel *et al.* (102) reported the recovery of a "herpes-like virus" from a dog kidney cell culture.

Since 1965 the CHV has been isolated from several cases of infant dog mortality (32, 37, 49, 58, 81, 91, 98). The isolation of CHV in the United States (12, 28, 65, 98, 101, 102), England (32, 90, 91), Japan (79), France (38), and Australia (49, 58, 81) indicates that this virus has a world-wide distribution.

In the past the primary importance of CHV as a pathogen has been in the disseminated disease produced in fetal and neonatal dogs. However, in common with herpesviruses of other animals, CHV has been shown to be an important pathogen of various organ systems in adult dogs. If one examines the herpesviruses of several of our domestic animals, a pattern of disease characteristic of this group of viruses can be observed. The pattern is one of fatal generalized disease of the fetus or the very young, mild upper respiratory infection and infection of the genital tract of older animals, and central nervous system infection, primarily, but not exclusively in the young. This pattern is seen with Herpesvirus hominis infections of man (99), H. bovis in cattle (11, 63, 70), equine herpesvirus 1 in horses (18, 19), and H. suis in swine (63).

The Effect of Temperature on the Canine Herpesvirus

The problem of why the CHV causes a fatal disease in neonatal pups while causing only relatively mild symptoms in adult dogs, has intrigued researchers since the CHV was

discovered. Andervont (1) working with H. hominis was the first to demonstrate that age was an important factor in the susceptibility of the host to infectious agents.

The fatal outcome of CHV infections of neonatal pups is also typical of herpetic infections of newborn infants (99), newborn calves infected with H. bovis (11, 18, 63), and H. suis infections of piglets (18, 63).

Johnson (62) proposed a cellular basis for the development of resistance with age in H. hominis infections of mice. He observed that peritoneal macrophages from neonatal and mature mice were equally susceptible to H. hominis, but that mature macrophages differed in their ability to release virus in that they would not infect other cells in contact with them, while infected neonatal mouse macrophages did infect other cells in contact with them. This phenomenon was termed a "barrier", but the mechanism was not determined. Carmichael and Barnes (25) were unable to show any difference in the release of virus from macrophages of neonatal or mature dogs.

Carmichael et al. (26), in an attempt to evaluate intrinsic cell resistance and other factors that could explain the greater virulence of CHV for the neonatal pup, were unable to attribute the sudden change from high susceptibility to high resistance, over a period of a few days, to macrophages or to the interferon system. These authors observed that there was no difference in "receptors" for

the CHV on cells of dogs of different ages. However, they were able to show that the multiplication of CHV in dog kidney cell cultures and in cultures of dog macrophages was markedly affected by the temperature at which viral replication was allowed to proceed.

The effects of temperature on the growth of CHV has been well documented (7, 8, 26, 76). Aurelian (7) was the first to observe that the multiplication of CHV was markedly affected by the temperature at which viral replication took place. The incubation temperature of CHV infected cell cultures that gave maximum yields of infectious virus was between 35 and 37 C (7, 25). This optimal range for viral multiplication is below the normal temperature, 38.9 C (102 F) (42), of the mature dog, however, this range of 35 - 37 C approximates the body temperature of neonatal pups raised at ambient temperatures between 21 and 27 C (25). CHV infected dog kidney and dog macrophage cultures incubated at 38 C (the normal rectal temperature for pups that have developed thermoregulatory control (47)) yielded approximately 10 percent as much virus as cultures incubated at 35 C (25). At 39.5 C virus yield was less than 0.1 percent of the maximum (25). The elevated temperatures affect viral replication specifically, since uninfected cells survive and multiply at 42 C (25).

The mechanism of inhibition of CHV multiplication at elevated temperatures has been studied and it appears that

the thermosensitive stage is late in the growth cycle (76, 103). Lust and Carmichael (76) observed that viral DNA and proteins were synthesized at 40 C and they suggested that the reason the formation of infectious virus was blocked at elevated temperatures was faulty maturation or assembly of the virus. In contrast, Stevens (103) observed that H. bovis viral specific proteins were synthesized at 42 C but synthesis of viral DNA could not be detected. He therefore concluded that the blocking of a temperature-sensitive step precludes the synthesis of viral DNA and therefore the formation of mature viruses. This author hypothesized that either the formation of a specific virus-induced DNA polymerase might be inhibited by heat, or that a step following viral uncoating, which involves a change in the infecting DNA so that it can be replicated, could be heat sensitive. Using temperature "shift down" experiments, Stevens was able to show that the blocking step occurred one hour after infection. In addition he showed that the virus induced an increased activity of a unique DNA polymerase at 37 and 42 C. Stevens concluded that the heat-sensitive step is concerned with an essential change either in the intracellular location or in the physical state of the infecting DNA molecule.

Studies of the thermal balance of new-born pups have shown that during the first 2 weeks of life the neonatal pup is unable to regulate its body temperature (34, 35, 47).

Crighton (34) summarized the situation in the neonatal pup by stating that "The newborn puppy in its usual environment is seen as a true homiotherm whose Lower Critical Temperature is so high that it is born into its own Zone of Hypothermia, and in order to maintain a steady high rectal temperature it substitutes for its lack of compensatory thermogenesis, compensatory thermal conduction." By the third to the seventh day of life compensatory heat production in the face of heat loss begins to overtake the role of thermal conduction and by the seventh day the pup is able to withstand temperatures down to 11 C (34). The shivering response has been observed in pups at 6 days of age (34); however, temperature homeostasis does not become fully operative until the third week of life (26).

Carmichael et al. (26) observed that during the first week of life, pups reared with their dams at a temperature of 21 C had body temperatures 1.7 to 4.4 C (3 to 8 F) lower than adult dogs. Knowing that neonatal pups have a relatively low body temperature, which they are unable to fully regulate until approximately the third week of life, and knowing that the CHV is very heat sensitive, these authors proposed that lower body temperature might be an important factor in the pathogenesis of CHV. Schmidt and Rasmussen (97) working with mice found them more resistant to H. hominis when reared at 37 C rather than 24 C. The lower percent of mortality was attributed to a lower concentration of virus

in the brains of these mice. Neonatal pups reared in a heated environment that caused elevation of their body temperature equal to that of an adult dog had increased survival rates following CHV infection and viral growth was reduced (26). Studies using 4-to 8-week-old dogs provided more evidence for the role of body temperature in modifying the symptoms, lesions, and viral multiplication of CHV in dogs (26). In these studies it was observed that dogs having artificially lowered body temperatures yielded virus from the liver, spleen, and kidneys, whereas no virus was isolated and lesions were absent in littermates whose body temperatures were not reduced. The fact that cells of non-neonatal dogs would support viral growth supported previous findings that viral multiplication of CHV was essentially equal in macrophages from 2-day-old and from 10-week-old pups.

Carmichael et al. (26) observed that the growth of CHV in mature animals seems to be restricted to the cooler portions of the body. These authors, using a thermistor needle-probe, measured the temperatures of the various external orifices and internal organs. The areas in which CHV has been identified as growing in the mature dog all have lower temperatures than the normal body temperature of the mature dog. For example, the temperature of the nasal cavity was found to be 3 to 4 C cooler than the normal body temperature.

The body temperature may also have an indirect effect on herpesvirus infections. Leucocytes have a definite function in destroying herpesvirus in the presence of immune serum (60). This together with the fact that there is a stimulatory influence of elevated temperature on phagocytic activity (55), suggests another mechanism by which body temperature may influence CHV infections.

The capacity for an inflammatory response is important in determining the pathogenesis of a CHV infection. Inflammatory reactions have not been observed around necrotic foci found in natural cases or in artificially infected pups that died of a CHV infection (28). In another study only pups that survived longer than 2 weeks had microscopic lesions that indicated that an inflammatory response was present (26). The febrile response is virtually absent until the pup is 2 to 3 weeks old (26).

Carmichael et al. (26) concluded that both regulation of temperature and the capacity for inflammatory and febrile responses are important factors that relate to the pathogenesis and recovery processes of CHV infections in young pups.

Herpesvirus Infections of Neonatal Pups

The CHV has been isolated from numerous tissues of neonatal pups which died of a disease characterized by disseminated focal necrosis and hemorrhage (22, 29, 91, 98, 105).

Susceptible pups acquire the infection through contact with infected dogs (57). Stewart et al. (105) have reported CHV infection in pups obtained by Caesarean section from apparently normal bitches. Thus, pups may be infected in utero via transplacental infection. Pregnant bitches inoculated intravaginally 2 weeks before whelping, have produced pups with fatal CHV infections (22). Carmichael (23) reported that it is probably more common for a litter of pups to be infected while passing through the birth canal of a bitch recently exposed to the virus than by in utero infection.

The gross pathological changes in CHV infected pups are similar in both natural and experimental cases (31). The lesions consist of hemorrhages and widespread focal necrosis that involve not only parenchymal and stromal cells but also blood vessels (23). The lesions are found in virtually all tissues (23), and by the fluorescent antibody technique virus has been demonstrated in virtually every tissue examined (113). However, lesions are especially noticeable in the liver, kidney, lungs, adrenal glands, intestines, and turbinates (57, 113). Changes in the kidneys are especially severe and the characteristic renal hemorrhages are considered of diagnostic significance (31). These lesions in the kidney consist of cortical necrosis and hemorrhages, which appear as circumscribed red areas on a dull gray background (23, 31). Cornwell and Wright (31)

stated that the lungs are usually edematous with frothy bloodstained fluid in the bronchi and trachea and there are multiple small hemorrhages scattered throughout the lobes. Hemorrhages are commonly found in the intestine, gastric mucosa and liver (31). The liver occasionally will have a few yellow foci on the surface (23).

Wright and Cornwell (115) reported that under experimental conditions, neonatal pups are highly susceptible to infection with CHV by the intranasal route. These authors noted that the main lesion was a focal necrotizing rhinitis which appeared as early as 2 days after infection in susceptible pups kept in contact with experimentally infected litter-mates. They concluded that the nasal mucosa was an important portal of entry for the CHV and that CHV gains access to the bloodsteam early in the course of the disease by extension of the necrotic process through the nasal epithelium and involvement of the blood vessels in the underlying lamina propria.

Clinical signs of central nervous system involvement have not been reported in pups naturally infected with CHV, but it has been shown that experimental inoculation by the intranasal, intracerebral, or intraperitoneal route will cause meningoencephalitis (87). Percy et al. (87) stated that, depending on the course of the disease, lesions may vary from discrete foci of neuronal degeneration, microgliosis, and perivascular cuffing, to diffuse hemorrhagic

meningoencephalitis characterized by necrosis, cavitation, and hemorrhage.

Percy et al. (85) have described the lesions in pups that have survived infection with CHV. These authors found that experimentally CHV-infected pups that subsequently recovered from the disease had focal granulomatous encephalitis, interstitial pneumonitis, and segmental renal necrosis with dysplasia. They also reported that pups which recovered from naturally occurring infections had segmental cerebellar and retinal dysplasia.

The gross and histopathologic findings of natural and experimental cases of CHV infections in neonatal pups have been well reviewed in the literature (24, 28, 84) and will not be discussed further.

Canine Herpesvirus Infection in Non-neonatal Dogs

The major emphasis of CHV research has been concerned with the disease it causes in the neonatal pup. However, recent isolations have been made from adult dogs with a variety of conditions and it appears that a better understanding of all the disease conditions CHV causes is needed before the complete impact of this virus on the canine population can be ascertained. CHV has been isolated from kidney cell cultures prepared from normal pups (101, 102), from dogs with upper respiratory tract disease (12, 67, 68), from a pup (2) and an adult dog with distemper (79),

from a dog with malignant lymphoma (65), from apparently healthy dogs (67), and from the genital tracts of dogs with a history of infertility, abortion, and stillbirths (90). Six-week-old pups which have had CHV inoculated into the conjunctival sac have shown signs of mild conjunctivitis with chemosis and serous ocular discharge (31).

The literature contains reports of negative results or mild disease resulting from experimental inoculations of CHV by various routes in the non-neonatal dog. Appel et al. (2) exposed 29 young beagle dogs to CHV by the oral-nasal route and reported either mild or inapparent respiratory infections. These authors also reported that oral-nasal and intravaginal inoculation of dogs older than 2 weeks of age caused only mild inflammation of the upper respiratory tract and vaginal mucosa respectively, and that viral growth was restricted to these tissues, except for occasional transient leukocyte-associated viremia. Huxsoll and Hemelt (57) reported that they were unable to produce clinical disease in susceptible 5 and 8-month-old dogs experimentally infected with CHV.

Canine respiratory infections are common in epidemic form wherever dogs are housed together. One respiratory condition which has been a problem in animal hospitals and other institutions where large numbers of dogs are housed together, is infectious canine tracheobronchitis, also termed "kennel cough." The outstanding sign of this disease

is a cough manifested by a short, dry "hack" or by a series of such coughs (23). Karpas et al. (68) reported the isolation of a herpesvirus from a group of dogs with infectious canine tracheobronchitis. From the same group of dogs, Karpas et al. (68) were able to isolate CHV from dogs showing no clinical signs characteristic of the disease. Karpas et al. (66) reported a variable clinical course of respiratory disease in experimental dogs inoculated intranasally. All the dogs had rhinitis to a variable degree and sneezed occasionally. Sporadic coughing could be caused in 2 dogs by palpating the trachea; another dog had a paroxysmal cough which lasted for 2 weeks. In another group of 8 dogs inoculated intranasally with CHV, they reported a spontaneous cough in 3 dogs (66). This report is in contrast to the findings of Appel et al. (2), who reported that 48 hours after inoculation with CHV by the oral-nasal route the dogs only had a mild rhinitis with a scant amount of serous nasal discharge. Appel et al. also reported that some dogs developed a mucoid nasal discharge after 3 to 4 days. Occasionally sneezing was observed in several of these dogs between the 4th and 8th days postinoculation. However, none of these dogs coughed, and coughing could not be induced by palpating the trachea. The gross lesions in these dogs consisted of hyperemia of the nasal turbinate mucosa and slight reddening of the posterior pharynx of dogs killed between the 2nd and 6th days postinoculation.

Lung lesions consisted of a moderate increase in the number of interstitial cells and there was some thickening of interalveolar septa. A few polymorphonuclear leukocytes and macrophages were seen, increasing the cellularity of alveolar walls.

Wright et al. (118) have reported the isolation of CHV from a neonatal dog in the United Kingdom. Experimental inoculation of this virus into susceptible 6-week-old pups did not cause any clinical symptoms. However, at post-mortem examination, they observed focal necrotizing pneumonia in the experimental pups. These authors suggested that CHV does not cause overt signs of respiratory disease, but like other herpesvirus infections, CHV might exist in the adult dog population in a latent state and is associated with respiratory disease only in times of stress.

Many other agents have been isolated from dogs with respiratory disease. Binn et al. (12) reported isolation of canine adenoviruses, a canine herpesvirus, and a SV-5-like virus from dogs with respiratory disease. The importance of a SV-5-like parainfluenza virus in respiratory disease of dogs has been supported by other investigators (3, 14, 33, 94). Appel et al. (4) reported that in their study only SV-5-like virus was isolated from dogs with a dry cough. These dogs were immune to canine distemper and infectious canine hepatitis. These authors also found that the SV-5-like virus in combination with Bordetella bronchiseptica and a

mycoplasma caused a harsh dry cough which was more severe than when the virus was used as the sole inoculum. However, SV-5 virus was not isolated from all cases of dogs with the cough. Similarly canine adenovirus type 2 has been isolated repeatedly from dogs with respiratory disease (12, 21, 39), and respiratory disease has been produced with this virus (107, 117). Two other viruses that have been suggested as possible etiological agents of infectious canine tracheobronchitis are canine distemper and reovirus type 1 (4).

There are numerous reports of isolations of mycoplasmas from dogs with respiratory disease (3, 5, 14, 52). However, clinical disease has never been produced with mycoplasma alone in dogs (4). Mycoplasmas have frequently been isolated from dogs without signs of disease (4).

In 1970, in a review of the current status of canine respiratory disease, Appel et al. (4) concluded that possibly more than one virus is involved in infectious tracheobronchitis and that the presence of potentially pathogenic bacteria including the mycoplasma, added to the severity of this disease.

The Pathogenicity of the Canine Herpesvirus for the Canine Genital Tract

Appel et al. (2) reported that dogs infected by the oral-nasal route with CHV developed a mild inflammation of the vaginal mucosa. Poste and King (90) in Britain described

the first isolation of a CHV from naturally-occurring infections of the genital tract of male and female dogs. These infections were associated with a clinical history of infertility, abortion and stillbirths. The CHV isolated from this outbreak of genital infections was used to produce the same clinical condition in one of 3 female dogs inoculated intravaginally. The lesions in both the natural and experimental cases consisted of vesicular lesions that became circular and "pock-like." The lesions were found in the vestibule, commonly in the region of the urethral orifice, but lesions were present on most surfaces of the vagina in severe cases. In the male dogs the lesions were on the preputial mucosa and on the penis at the preputial reflection. Poste and King (90) recognized and reported the similarity of the lesions they observed to those reported previously in cases of so called "dog pox" (20, 72). The etiology of "dog pox" is unknown although infectious agents have been implicated (20). It has been reported that in addition to the genital lesions of primary "dog pox," the gum margins, gastrointestinal tract, anus, and conjunctiva were also affected on occasion (20). Support for the possibility that the etiological agent of "dog pox" is a herpesvirus is the fact that these sites of infection described above are known predilection and replication sites for herpesvirus in several species (88).

Several interesting features of the outbreak reported by Poste and King (90) are important not only to the understanding of the disease CHV causes in the genital tract of dogs but also to the epizootiology of the CHV complex in dogs. They reported that the CHV genital infections were transmitted by sexual contact and they also demonstrated the CHV in the genital tract of female dogs. These findings explained how the infection of the neonatal pup occurs during its passage through the birth canal. The reports also contained the first evidence that latency and recurrent infections may occur with CHV. Other workers (2, 31, 66, 105) have suggested recurrent and latent CHV infections and these phenomena are well-known features of herpesvirus infections of some other species of animals (88).

Poste (89) in a subsequent report, in which he characterized the CHV that was isolated from the genital tract of dogs (90), stated that although CHV-BR (Poste strain) is serologically very similar to the CHV strain F-205 isolated by Carmichael et al. (29), it differs from this strain by virtue of the CPE in DKC. CHV-BR causes the formation of polykaryocytes that have not been reported for other strains of CHV (89). Poste (89) also reported that the incidence of nuclear inclusions in DKC infected with the CHV-BR strain was considerably higher than that reported for other CHV strains. He also stated that these differences may reflect significant differences between the CHV strains but it is

possible that the site of virus isolation from the body might influence the type of CPE. Dowdle et al. (41) have observed an association of antigenic type of H. hominis with the site of viral recovery in man. They reported that there were 2 antigenically distinct groups of H. hominis which they designated type 1 and 2. All 42 strains which they identified as type 2 were isolated from herpetic infections related directly or indirectly to the adult genital tract, whereas, type 1 was isolated from sites other than the genital tract. Other workers have identified herpesviruses which are variants of a strain (45) or two separate strains of the same virus (111) on the basis of CPE induced. It has been reported that for the most part, H. hominis variants based on plaque morphology of CPE on various tissue culture systems are antigenically identical to the parent strain (80).

Immunological Aspects of the Canine Herpesvirus

The antibody response to CHV in adult dogs has been studied by Karpas et al. (67). These workers found that the levels of neutralizing antibody in naturally-infected dogs were variable, with negative titers possibly being a reflection of inadequate exposure to the CHV for the neutralizing antibodies to develop. Experimentally-infected dogs developed significant titers of neutralizing antibody after intranasal inoculation. Antibody titers were detected as early as 17 days after initial inoculation and they in-

creased to a peak at one month. Beyond one month the titers gradually decreased. Neutralizing antibody titers to CHV have been reported as high as 128 (67). Different techniques used to determine neutralizing antibody may cause variation in reported titers from different laboratories. It has been reported that the constant serum-varying virus (alpha) procedure is the most sensitive method (24). The addition of guinea-pig complement to the virus-serum dilutions has been reported to increase CHV neutralizing antibody titers two-to-eightfold (24). Complement-requiring neutralizing antibody to H. hominis in man appears one week after infection and reaches a considerable level before rises of complement fixing and non-complement-requiring neutralizing antibody (119). The complement-requiring neutralizing antibody is used in man for early detection of herpetic infections which are difficult to identify otherwise, since a large portion of the population has circulating antibodies to herpesviruses (119). There are no extensive serological surveys reported to indicate the extent of CHV in the canine population. However, Spertzel et al. (102) reported that antibody to CHV had been found in three lots of commercial canine globulin. The use of complement-requiring neutralizing antibody possibly could be important in identifying early cases of CHV antibody.

The relationship between neutralizing CHV antibody titers and immunity has not been established in adult dogs, but it

has been reported that maternal antibody will protect neonatal pups from the disease but not from the infection (24, 57). It has been suggested that it is CHV maternal antibodies that protect litters of pups born to bitches that have had a previous litter die of a CHV infection (57). Newborn pups that suckled from bitches with neutralizing antibody titers of 1:4 or greater showed no signs of illness after being inoculated orally or intraperitoneally with CHV (24). Although any apparent illness was absent, virus was recovered from the nasal, pharyngeal and rectal specimens taken from these pups.

Carmichael (24) suggests that neutralizing antibody titers are not always a good indication of resistance because neutralization of CHV by antibody is probably a poor indication of the biological activity of antibodies.

MATERIALS AND METHODS

Cell Lines

MDCK cells

The Madin-Darby canine-kidney (MDCK) cell line was used for propagation of all CHV and canine adenovirus, for viral isolation, and for serum-virus-neutralization tests (SVN). The MDCK cells were propagated at 37 C in 250 ml plastic tissue-culture flasks.[1] The growth medium consisted of minimum essential medium (Eagle) with Earle's salts and L-glutamine plus 10 percent bovine serum and 0.11 percent sodium bicarbonate. Monolayers of cells were trypsinized with 0.2 percent trypsin-versene (see preparation next page), diluted 1 to 3 in growth medium and dispensed into disposable glass tissue-culture tubes,[2] plastic tissue-culture plates,[3] and Leighton tissue-culture tubes.

Low passage DKC cells

Dog kidney cells (DKC)[4] between the third and sixth passages were propagated using the same growth medium as

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[1]Falcon Plastics, Los Angeles, California.

[2]Becton, Dickinson and Co., Rutherford, New Jersey.

[3]Linbro Chemical Co., New Haven, Connecticut.

[4]Obtained from Dr. Arnett Matchett, U. S. D. A., Biologics Division, Ames, Iowa.

was used for the MDCK cells. These cells were grown in glass tissue-culture tubes and were only used to compare the sensitivity of DKC and MDCK cells to CHV-270.

Maintenance Medium

Minimum essential medium (MEM) (Eagle) with Earle's salts and L-glutamine plus 2 percent bovine serum and 0.11 percent sodium bicarbonate was used for the maintenance of the cell-culture monolayers and for cell washing.

Water

The water used in all procedures and solutions was glass distilled and deionized. It contained less than 0.5 µg/ml of sodium chloride equivalents as measured with a conductivity meter.[1]

Trypsin-Versene Solution

A trypsin-versene solution was used to remove the DKC and MDCK cells from the surface of the 250 ml plastic flasks. The formulation was as follows:

Trypsin[2] (1:250)	2.0 g
Na Cl	8.0 g
KH_2PO_4	0.2 g
Na_2HPO_4	1.15g
H_2O	1.0 liter
Ethylenediamine Tetraacetic acid Tetra sodium salt	1.0 g

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[1]Barnstead Company, Boston, Massachusetts.

[2]Difco Laboratories, Detroit, Michigan.

Pucks Saline Solution G

Pucks saline solution G (92) was used as the diluent for all virus dilutions. The formula for this solution is as follows:

Water	1.0 liter
Glucose	1.1 g
Phenol red	5.0 mg
Na Cl	8.0 g
K Cl	0.4 g
$Na_2HPO_4 \cdot 7H_2O$	0.29g
K H_2PO_4	0.15g
Mg $SO_4 \cdot 7H_2O$	0.15g
Ca $Cl_2 \cdot 2H_2O$	16.0 mg

Viruses

Canine herpesvirus, strain F-205

Canine herpesvirus strain F-205[1] at the fifth passage level on DKC was propagated on MDCK cells in 250 ml tissue-culture flasks. The cell-culture fluid containing virus and cells was harvested when the CPE was nearly complete. Viral suspensions were frozen in liquid nitrogen and thawed to release the virus from the cells, centrifuged at 115 x g then stored at -90 C. CHV strain F-205 at the second passage level on MDCK was used in the cross-neutralization test with CHV-270 and for comparison of the CPE produced by CHV strain F-205 and CHV-270.

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[1]Obtained from Dr. L. E. Carmichael, Cornell University, Ithaca, New York.

Canine herpesvirus isolate 270 (CHV-270)

CHV-270[1] was isolated by Dr. A. Karpas from a clinical case of infectious canine tracheobronchitis. This virus had been passed 3 times on MDCK when received in our laboratory. The virus was passed further on MDCK cells, harvested, and stored at -90 C. CHV-270 at the 4th and 5th passage levels was used to inoculate dogs in experiments 1 through 4 and experiments 6 through 8. CHV-270 was also used as the challenge virus in experiment 5 and as the antigen in the preparation of the anti-CHV rabbit serum.

Temperature-attenuated canine herpesvirus isolate 270 (CHV-33)

CHV-33[1] is the CHV-270 isolate which when received had been passed 80 times on MDCK at 33 C. This virus was passed to the 91st passage level on MDCK at 33 C, harvested and stored as described for CHV strain F-205. CHV-33 was used at the 83rd passage level to inoculate the dogs in experiment 5.

Canine adenovirus, A26/61 strain

The canine adenovirus strain A26/61[2] was isolated by Ditchfield et al. (39) from experimental surgery dogs experiencing "kennel cough." This virus was received after 10

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[1]Obtained from Dr. G. P. Shibley, Haver-Lockhart, Shawnee Mission, Kansas.

[2]Obtained from Dr. L. Swango, Auburn University, Auburn, Alabama.

passages on DK cells, passed once on MDCK, harvested and stored as described previously. Canine adenovirus, strain A26/61 was used in a SVN test to check for the presence of canine adenovirus antibodies in serums randomly selected from dogs in experiments 4, 5 and 7.

Nutrient Agar-Overlay Medium

A double concentration of Eagle's basal medium (BME) with Earle's salts supplemented with 10 percent bovine serum and 0.22 percent sodium bicarbonate was added to an equal volume of 1.8 percent "Ionagar" #2.[1] Just prior to using this agar-overlay medium in the 35 x 10 mm wells of plastic tissue-culture plates, 10,000 units of penicillin and 10,000 µg of streptomycin were added to each 100 ml of the medium. The final concentration of each component was 0.9 percent agar, 5 percent bovine serum, and 0.11 percent sodium bicarbonate.

Neutral-Red Agar-Overlay Medium

When virus plaques appeared in the cell monolayers grown in plastic tissue-culture plates, a second agar-overlay was added to differentiate the plaques. This agar-overlay medium contained 1 percent "Ionagar" #2[1] and 10 mg per 100 ml of neutral red. After adding this second agar-overlay, the plates were placed in the dark for 8

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[1]Oxoid marketed by Colab Laboratories, Inc. Chicago Heights, Illinois.

hours to allow vital staining to occur. The plaques were then counted.

Virus Titration

Tenfold serial dilutions of the virus to be titrated were made in saline G. One half ml of each dilution of virus was inoculated into each of two 35 x 10 mm wells of a plastic tissue-culture plate, containing a 4-day-old monolayer of MDCK cells. The virus was allowed to adsorb for 2 hours at room temperature before being aspirated. The inoculated cell monolayers were then overlayed with 2 ml of nutrient agar-overlay medium per well. The plates were incubated at 37 C until plaques became visible. To aid in the differentiation of plaques the neutral-red agar-overlay was added. The plaques were counted and the titers of the virus were expressed in plaque-forming units (pfu) per ml of virus.

Preparation of Anti-CHV Rabbit Serum

The CHV antigen was prepared by inoculating a 4-day-old MDCK cell culture with CHV-270. When approximately 75 percent of the cells showed CPE, they were scraped from the surface of the flask and suspended in cell-culture medium. To release the viral antigen from the cells, the suspension was twice frozen in liquid nitrogen and thawed. In order to remove the cellular debris the suspension was centrifuged

at 2,000 x g for 30 minutes and the supernatant CHV antigen was then stored at -90 C.

Two young New Zealand white rabbits were used in preparation of the antiserum. For intravenous (IV) injection, undiluted CHV antigen was thawed and used immediately. The lateral ear vein was used for IV inoculations. For intradermal (ID) inoculations of the CHV antigen, the antigen was mixed with equal parts of Freund's complete adjuvant.[1] An emulsion of the antigen and adjuvant was prepared by adding 0.5 ml of antigen, drop by drop, to the adjuvant and agitating vigorously. The ID inoculations were made along the back of the rabbit, 0.2 ml at each site. Each rabbit was inoculated IV with 1 ml of the undiluted virus on days 1, 2, and 4 and ID with the emulsion on days 1, 7, 14, and 21. Both rabbits were bled on days 28 and 35. The blood was collected from the heart, allowed to clot at room temperature for 1 hour, refrigerated at 4 C overnight and then centrifuged at 275 x g to separate the serum from the clot. The serum was then removed from the clot, heat inactivated for 1 hour at 56 C, and stored at -20 C.

Serum-Virus-Neutralization Tests

The serum-virus-neutralization (SVN) test was used to determine antibody titers to CHV and canine adenovirus. Twofold dilutions (to a dilution of 1/512) of the serum to

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[1]Difco Laboratories, Detroit, Michigan.

be tested were made in saline G. Virus which had been previously titrated was diluted so that the final concentration would contain approximately 100 pfu per well. Equal amounts of virus and serum were mixed and incubated at room temperature for 1 hour. One half ml of each dilution of the serum-virus mixture was then inoculated into each of two 35 x 10 mm wells of a plastic tissue-culture plate, containing a 4-day-old monolayer of MDCK sells. The virus was adsorbed and then aspirated as described for virus titration. Two ml of nutrient agar-overlay was added to each well and when plaques appeared 2 ml of a neutral-red agar-overlay was added to aid in the counting of the plaques. The antibody titers were expressed as the reciprocals of the serum dilutions which produced 50 percent reduction in the number of viral plaques when compared with the number of plaques in the control plates.

Special Staining Procedures

MDCK cell-cultures grown on coverslips in Leighton tubes were used to propagate CHV strain F-205 and CHV-270. When between 25 and 50 percent of the cells showed CPE the coverslips were removed and stained as described below.

Acridine orange staining procedure

The acridine orange staining procedure (36) was used in an attempt to differentiate between the number of intranuclear inclusion bodies produced by the CHV-270 and

the CHV strain F-205. Acridine orange stained cell cultures were examined using an ultraviolet microscope.

Hematoxylin-phloxin-safran staining procedure

The hematoxylin-phloxin-safran staining procedure (110) was used to study the difference between the types of CPE produced by the CHV-270 and the CHV strain F-205.

Nuclear Feulgen reaction

The nuclear Feulgen reaction (74) was used to stain MDCK cell cultures infected with CHV-270 and CHV strain F-205, in an attempt to demonstrate a difference in the number of intranuclear inclusion bodies produced by these two viruses.

Blood Parameters

One ml of blood was withdrawn from the cephalic vein of dogs and collected in Vacutainer tubes[1] containing ethylenediaminetetraacetic acid. The packed cell volume (PCV) of the blood samples was determined using the micro-hematocrit method (96). White blood cell (WBC) and red blood cell (RBC) counts were determined using a Coulter Counter[2]. The hemoglobin concentration of the blood samples was measured using the spectrophotometic method (96).

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[1]Becton, Dickinson and Co., Rutherford, New Jersey.

[2]Coulter Electronics Co., Hialeah, Florida.

Collection of Specimens

Cotton-tipped applicators (cotton swabs) were used to obtain specimens for viral isolation from the nasal cavity, conjunctival sac, pharynx, and genital tract (vagina or sheath). The cotton swab was inserted into the desired site and gently rotated so that as much of the swab as possible contacted the tissue. The cotton swabs were then put into tubes containing 2 ml of antibiotic "cleaning fluid" (formulation next section). After all the specimens were collected the cotton swabs were pressed against the side of the tubes to remove as much of the fluid as possible. The swabs were then discarded and the fluid was used for viral and mycoplasmal isolations. Specimens for viral isolation were obtained on days 2, 4, 6, 9, 12, 14, 16, and 20 or at the time of death. Blood samples for viral isolation were taken from the cephalic or the jugular vein. One ml of blood was added to 2 ml of antibiotic "cleaning fluid" (described later) for this purpose.

For serum-virus-neutralization (SVN) tests, blood was withdrawn from the jugular vein before inoculation and on days 7, 14, 21, and 28. After standing at 4 C overnight the blood was centrifuged at 275 x g to separate the serum from the clot. The serum was removed and stored at -20 C until it was used in the SVN test.

Antibiotic "Cleaning Fluid"

For the primary isolation of viruses, all specimens were suspended in 2 ml of cleaning fluid prepared in the following manner:

For 600 ml of cleaning fluid:

585 ml BME containing 0.11 percent $NaHCO_3$

600,000 units crystalline penicillin and 600,000 µg dihydrostreptomycin in 3 ml of distilled water

12,000 µg Fungizone in 12 ml of distilled water

The final concentration of antibiotics was 1,000 units of penicillin, 1,000 µg/ml of streptomycin, and 20 µg/ml of Fungizone.

Viral Isolation

After standing at room temperature in antibiotic "cleaning fluid" for 2 hours those specimens that contained a large amount of particulate material were cleared by centrifuging at 115 x g for 10 minutes. Blood samples, like other specimens, were placed in 2 ml of viral cleaning fluid, frozen in liquid nitrogen, and thawed to disrupt the red blood cells. Medium from tubes containing 4-day-old MDCK cell cultures was poured off and 0.25 ml of the specimen was pipetted into each of 2 tubes. The specimens were allowed to adsorb at room temperature for 2 hours. Each tube was rinsed with 1.0 ml of maintenance medium. One ml

of maintenance medium was then added and the specimens were incubated at 37 C. Each tube was examined every other day for the presence of CPE. When 75 percent of the monolayer of cells showed CPE, the specimens were stored at 4 C for subsequent viral identification.

Viral Identification

The first and last viral isolation from each site sampled, i.e. the nasal cavity, was identified by a tube serum-virus-neutralization test. Four tubes containing 4-day-old MDCK cell cultures were used to identify each isolate. The tubes were inoculated and virus adsorbed as previously described for viral isolations. Two of the four tubes from each isolation received maintenance medium containing 10 percent anti-CHV rabbit serum whereas the other two tubes received maintenance medium containing 10 percent normal rabbit serum. Each tube was examined daily for evidence of CPE. Positive CHV identification was made on the basis of partial or complete neutralization by the positive CHV antiserum.

M-96 Mycoplasma Medium

M-96 mycoplasma medium was used for the propagation of all mycoplasmas. The formulation of this medium is as follows: (Dr. M. Frey, Unpublished data. Veterinary Medical Research Institute, Iowa State University, Ames, Iowa.)

	Per liter	
Peptone CS[a]	4.0	g
Peptone B[a]	2.0	g
Peptone G[a]	2.0	g
Yeast autolysate[a]	2.0	g
Yeast extract[a]	2.0	g
Na Cl	5.0	g
K Cl	0.4	g
$Mg\ SO_4 \cdot 7H_2O$	0.2	g
Catalase	0.001	g
Hepes buffer	3.5	g
L-arginine H Cl	0.06	g
L-glutamine	0.09	g
DNA*	0.02	g
Eagle's MEM vitamin solution 100x[b]	10.0	ml
Cholesterol emulsion**	2.0	ml
Glycerol	0.15	ml

[a]Pfizer Diagnostics, Chicago, Illinois.

[b]International Scientific Industries, Cary, Illinois.

*Two ml of a 1 percent solution of DNA (not highly polymerized) in water buffered to pH 7.2.

**Cholesterol emulsion (Method of Edward and Fitzgerald) (43): Two hundred mg of cholesterol was put in a sterile petri dish and dissolved in diethyl ether. The ether was allowed to evaporate and the process was repeated.

The sterile, recrystallized cholesterol was dissolved in 6-8 ml warm 95 percent ethyl alcohol.

The alcohol solution was drawn into a prewarmed glass Luer-lock syringe fitted with an 18 or 20 gauge needle.

Two hundred ml of distilled, deionized water was boiled to drive off the absorbed CO_2. The water was then cooled to 90 C and the cholesterol was injected beneath the surface of the water.

The solid components were dissolved in 400 ml of water and the liquid components were added to this solution. Water was added to bring the volume to 500 ml. To prepare M-96 liquid medium an equal volume of water was added to the above solution. To prepare M-96 agar a double concentration of molten agar[1] (1.5 percent) was added to 500 ml of double-strength M-96 liquid medium. The M-96 liquid mycoplasma medium was supplemented with 10 percent heat-inactivated (56 C for 30 minutes) rabbit serum. For the primary isolation of mycoplasmas, 1,000,000 units/liter of penicillin and 5 g/liter of thallium acetate were added to inhibit the growth of all bacteria except for the mycoplasmas. M-96 mycoplasma agar was also supplemented with 10 percent heat-inactivated rabbit serum.

Mycoplasmal "Cleaning Fluid"

Equal volumes of specimen, suspended in antibiotic "cleaning fluid", and mycoplasmal "cleaning fluid" were mixed and allowed to stand at room temperature for 90 minutes before being used as inoculum. To prepare the mycoplasma "cleaning fluid" 500,000 units of penicillin and 200 mg of thallium acetate were added to 100 ml of sterile water.

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[1]Difco Laboratories, Inc., Detroit, Michigan.

Isolation of Mycoplasma

After standing at room temperature for 90 minutes 0.5 ml of each mycoplasma specimen was added to 4.5 ml of mycoplasma medium containing 2 mg thallium acetate and 1,000 units of penicillin. After 48 hours incubation at 37 C, 0.5 ml of each culture was transferred to 4.5 ml of uninhibited mycoplasma medium. This step was repeated after another 48 hours. Cultures that showed evidence of mycoplasmal growth such as turbidity when the contents of the tube was swirled, were inoculated onto mycoplasma agar plates. One drop of the culture was placed on the agar and the plate was tilted to allow the inoculum to spread. The plates were incubated at 37 C in a CO_2 incubator containing 5 percent CO_2 until the mycoplasma colonies appeared, which was usually after 48 hours. Mycoplasma colonies were identified by their typical "fried egg" appearance when observed under low power magnification.

Bacteriological Media

Tryptose blood agar

Tryptose blood agar base with 5 percent citrated bovine blood and 1.8 percent agar with 1 percent proteose-peptone #3 added was used in the isolation and identification of *B. bronchiseptica*.

MacConkey's agar

MacConkey's agar fortified with 1 percent dextrose was

also used in the isolation and identification of B. bronchiseptica.

Sugar base media

Sugar base media used in the isolation and identification of B. bronchiseptica contained 0.5 percent of the dextrose or lactose in tryptose broth. The formula for the tryptose broth used is as follows:

Bacto-Beef extract[1]	3.0 g
Bacto-Yeast extract[1]	3.0 g
Tryptose	15.0 g
Na Cl	5.0 g
Andrades indicator	10.0 ml
Water	Q. S. 1.0 liter

Andrades indicator

Andrades indicator used in the sugar base media was formulated as follows:

Acid fuchsin	0.5 g
NaOH (1N)	16.0 ml
Water	Q. S. 100.0 ml

The ingredients are combined and allowed to stand for 10 days after which the solution is filtered.

Litmus milk

Litmus milk was used in the identification of B. bronchiseptica. The litmus milk was prepared as follows:

Bacto-litmus[1]	0.15 g
Bacto-skim milk powder[1]	30.0 g
Distilled water	Q. S. 300.0 ml

[1]Difco Laboratories, Inc., Detroit, Michigan.

The litmus milk was dispensed into tubes with cotton stoppers so that the characteristic blue ring formed during the alkalinization process.

Bacterial Isolation and Identification

Bordetella bronchiseptica was of primary concern as a resident bacterium in the experimental dogs because it has been incriminated as a primary and secondary etiological agent in canine respiratory disease. Colonies suspected of being B. bronchiseptica (on the modified MacConkey's agar plates) were selected and inoculated into tryptose broth. After 24 hours incubation a drop of this culture was heat fixed on a glass slide and stained with Gram's stain. Any cultures that were gram-negative pleomorphic rods showing coccoid or bacillary forms ranging from 0.5 to 1.5 μm were examined further by inoculating differentiating media.

To make positive identification the suspected cultures were inoculated into litmus milk, Simmons' citrate agar[1], glucose broth, lactose broth, and urea broth.[1] B. bronchiseptica cultures hydrolyze urea within 12 to 24 hours of incubation. Citrate is utilized within 1 to 2 days, and litmus milk and dextrose are alkalinized slowly. Lactose is not utilized by B. bronchiseptica.

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[1]Difco Laboratories, Inc., Detroit, Michigan.

Experimental Dogs

Dogs used in experiments 1 through 4 were 6-month-old Beagle dogs bred and raised in the closed dog colony of the Veterinary Medical Research Institute (VMRI) at Iowa State University. The dogs used in experiment 6 were 16-week-old Beagle dogs that were also obtained from the VMRI colony. Ten-week-old Beagle dogs obtained from a commercial dog breeder were used for experiment 7. Beagle dogs were obtained from both the VMRI colony and a commercial dog breeder for experiment 5. Dogs 19 through 26 and dogs 29, 30, and 32 were obtained from the VMRI colony at 16 weeks of age for experiment 5, while dogs 27, 28, 31, 33, and 34 were obtained from the commerical colony at 12 weeks of age. The 4 dogs used in experiment 8 were 12-week-old mixed breed dogs from a litter of pups raised on a local farm.

Preinoculation Examination

All dogs were examined for signs of disease prior to experimentation. The pharyngeal area, nasal cavity, conjunctival sac, and vagina or penis of each dog was swabbed with a cotton swab. Each swab was placed in 1 ml tryptose broth and used for inoculation of blood agar and MacConkey's agar for isolation of B. bronchiseptica. Of the remaining specimen 0.25 ml was added to 0.25 ml mycoplasma "cleaning fluid" and 0.25 ml was added to 2 ml antibiotic "cleaning fluid" for mycoplasma and virus isolation respectively.

Histological Procedures

Tissues collected at necropsy were fixed in 10 percent buffered formalin for 4 days. Sections of nasal turbinate and ribs were decalcified using Decal[1] for 12 hours then washed in running tap water for 6 hours. All tissues were dehydrated in graded ethanol solutions, cleared in xylene, and embedded in paraffin. Sections were cut at 6 μm and mounted with Permount[2] mounting medium on glass slides. All sections were stained with Harris's hematoxylin and eosin Y (H and E). These procedures were carried out as described in the Armed Forces Institute of Pathology's Manual of Histology and Special Staining Techniques (74).

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[1]Scientific Products Division, Hospital Supply Corp., Evanston, Illinois.

[2]Fisher Scientific Company, Fair Lawn, New Jersey.

EXPERIMENTAL PROCEDURES AND RESULTS

Laboratory Comparison of CHV-270 and CHV F-205

The results from cross SVN tests using CHV strain F-205 and CHV-270 viruses and CHV-270 rabbit antiserum and CHV strain F-205 antiserum[1] are listed in Table 1. The antiserum dilutions which neutralized CHV F-205 were identical while the antiserum dilution which neutralized CHV-270 was greater for CHV-270 than it was for CHV F-205 but the difference was not significant.

Table 1. Cross serum-virus-neutralization tests between CHV-270 and CHV F-205

		Antiserum	
		CHV-270	CHV F-205
Virus	CHV F-205	64[a]	64[a]
	CHV-270	64[a]	32[a]

[a]Antibody titers are expressed as the reciprocal of the highest dilution of antiserum that caused a 50 percent reduction in plaques in comparison to controls.

Microscopic examination of MDCK cell cultures infected with CHV strain F-205 or CHV-270 and stained with hematoxylin-phloxin-safran or with H and E revealed similar CPE. The affected cells in both infections were rounded and clumping of the nuclear chromatin was observed. Intranuclear inclusion bodies that developed in MDCK cells

[1]Obtained from Dr. L. E. Carmichael, Cornell University, Ithaca, New York.

infected with CHV F-205 were similar in number and in morphology to inclusions observed in MDCK cells infected with CHV-270. The inclusion bodies were eosinophilic and varied in size, sometimes completely filling the nucleus.

The sensitivity of MDCK cells and DKC to infection with CHV-270 and CHV strain F-205 was determined. Neither cell type was significantly more sensitive to either virus. Due to the ease of working with the MDCK cells in the laboratory, they were used throughout these experiments as the laboratory host system.

Adenovirus A26/61 Antibodies in CHV-Infected Dogs

Antibody titers to adenovirus A26/61 were determined by the SVN plaque-reduction method. The antibody titers to adenovirus A26/61 of several dogs infected with CHV-270 are listed in Table 2.

Table 2. Adenovirus A26/61 antibody titers of selected dogs

Dog No.	Experiment No.	Day Sampled	Titer[a]
14	4	28	<2
23	5	56	<2
25	5	56	<2
27	5	56	<2
42	7	7	<2
43	7	7	<2

[a]Antibody titers in this table and all subsequent tables are expressed as the reciprocal of the highest dilution of antiserum that caused a 50 percent reduction in plaques in comparison to controls.

Results of Preinoculation Examinations

Dogs in all experiments were found to be healthy and all had temperatures between 37.8 and 39.2 C (100-102.5 F). No viruses were isolated from these dogs during the preinoculation examinations. Many mycoplasmas were isolated from the various sites sampled as indicated in Table 3. Bordetella bronchiseptica was isolated from several dogs as indicated in Table 4, however, there was no correlation between the isolation of B. bronchiseptica and the incidence of clinical symptoms.

The ubiquity of mycoplasmas in dogs and the fact that no mycoplasma pathogens have been identified for dogs makes it difficult to evaluate the importance of their isolation. In this study the mycoplasmas were considered as part of the normal microflora of the dog.

Experiment 1:
Pathogenicity of CHV in Dogs Inoculated Intranasally

The purpose of this experiment was to test the pathogenicity of CHV-270 when inoculated intranasally into dogs. Dogs 5, 6, 7 and 8 received 5.3×10^5 pfu of CHV-270, 0.5 ml in each naris, while dogs 1, 2, 3, and 4 received 1.5 ml of MDCK cell-culture fluid in each naris on day zero. Blood, pharyngeal, nasal and genital specimens were obtained on days 2, 4, 6, 9, 12, and 16 for virus isolation. Conjunctival samples were obtained on days 9, 12, and 16 only. Blood samples for hematology were obtained on days 2, 4, 6, 9 and 12, and blood samples for serological tests were obtained on days zero and

Table 3. Results of preinoculation examinations of experimental dogs for mycoplasmas

	Dog No.	Oral	Nasal	Genital	Conjunctival
Experiment 1	1	+[a]	+	+	N.D.[b]
	2	+	0[c]	0	N.D.
	3	+	0	0	N.D.
	4	+	0	+	N.D.
	5	+	0	0	N.D.
	6	+	0	0	N.D.
	7	+	0	0	N.D.
	8	+	0	0	N.D.
Experiment 2	Used control dogs of experiment 1, dog # 1, 2, 3, and 4.				
Experiment 3	Used control dog of experiment 2, dog #3.				
Experiment 4	14	+	0	0	0
	15	+	0	0	0
	16	+	0	0	0
	17	+	+	0	0
	18	+	0	0	0

[a]Positive isolation of mycoplasma.

[b]Not Done.

[c]No mycoplasma isolated.

Table 3. Continued

	Dog No.	Oral	Nasal	Genital	Conjunctival
Experiment 5	19	+	0	0	0
	20	0	0	0	0
	21	0	0	0	0
	22	0	0	0	0
	23	+	+	0	0
	24	+	0	0	0
	25	+	0	0	0
	26	+	+	+	0
	27	+	+	+	+
	28	0	+	+	+
	29	+	+	0	+
	30	+	+	0	0
	31	+	+	0	+
	32	0	0	0	0
	33	+	0	0	0
	34	+	0	0	0
Experiment 6	35	+	0	0	0
	36	+	0	0	0
	37	0	0	0	0
	38	+	0	0	0
	39	+	0	0	0
	40	+	0	0	0

Table 3. Continued

	Dog No.	Oral	Nasal	Genital	Conjunctival
Experiment 7	41	+	0	0	0
	42	+	+	0	0
	43	+	0	0	+
	44	+	0	0	0
	45	+	+	0	0
	46	+	0	0	0
	47	0	+	0	0
	48	+	+	0	+
Experiment 8	49	+	+	0	0
	50	0	+	0	0
	51	0	+	0	0
	52	0	0	0	0

Table 4. Results of preinoculation examinations of experimental dogs for *Bordetella bronchiseptica*

Dog No.	Experiment No.	Pharynx	Nasal cavity
2	1	0[a]	+[b]
4	1	0	+
27	5	0	+
28	5	0	+
29	5	0	+
30	5	0	+
31	5	0	+
36	6	+	0
37	6	+	0
41	6	0	+
43	7	0	+
45	7	0	+
47	7	+	0

[a]No *B. bronchiseptica* isolated.

[b]Positive isolation of *B. bronchiseptica*.

28.

Results of virus isolation are recorded in Table 5. Virus was isolated for 9 days from the infected dogs. The isolations from the genital tract and from the conjunctiva indicated that the virus spread to these sites. No viremia was detected as indicated by the lack of viral isolations from the blood specimens, however, the sampling interval may have been too great to detect a viremia. All 8 dogs had negative antibody titers before inoculation as indicated in Table 6. The control dogs remained negative and the dogs that were infected developed antibody titers of 32[1] to 64.

The results of the hematologic tests, recorded in Table 7, revealed few changes from the normal. The packed cell volume, white blood cell count, red blood cell count and the hemoglobin content all remained within normal ranges (96) except for 3 RBC counts that dropped slightly below the normal range.

The clinical signs, which are recorded in Table 8, were primarily associated with the eyes. Excessive lacrimation and conjunctivitis were often accompanied by an accumulation of serous exudate at the medial canthus of the eye. Dog number 2 was observed sneezing on day 2 postinoculation. The temperature of all dogs remained within normal limits throughout the 28-day experiment.

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[1]All antibody titers in this and all subsequent experiments are expressed as the reciprocal of the highest dilution of antiserum that caused a 50 percent reduction in plaques in comparison to the controls.

Table 5. Experiment 1: CHV reisolated from dogs after intranasal inoculation of CHV-270

Dog No.	Days sampled					
	2	4	6	9[a]	12	16
5	N[b]	NP	NP	NP	0[c]	0
6	NPG	NP	NP	0	0	0
7	NPG	NPG	NPG	NPC	0	0
8	NP	NP	NPG	NPC	0	0

[a]Conjunctival sac swabbed only on days 9, 12 and 16.

[b]N, P, G and C represent positive CHV isolations from the nasal cavity, pharynx, genital tract and conjunctival sac, respectively.

[c]No CHV isolated.

Table 6. Experiment 1: CHV antibody titers of dogs after intranasal inoculation of CHV-270

Dog No.[a]	Antibody titers	
	Day 0	Day 28
1	<2	<2
2	<2	<2
3	<2	<2
4	<2	<2
5	<2	64
6	<2	64
7	<2	64
8	<2	32

[a]Dogs #1, 2, 3 and 4 = uninfected control dogs.

Table 7. Experiment 1: Blood parameters of dogs after intranasal inoculation of CHV-270

Dog No.	Day Sampled	Blood parameters			
		PCV[a]	WBC[b]	RBC[b]	Hemoglobin (mg %)
1	2	49	10,700	6.2	16.9
2	2	44	10,600	6.2	15.6
3	2	44	15,500	5.8	15.6
4	2	43	12,300	5.9	15.2
5	2	50	10,900	6.5	16.4
6	2	39	6,300	5.4	13.6
7	2	41	8,900	5.1	13.6
8	2	43	15,300	5.9	14.0
1	4	46	12,200	6.0	16.1
2	4	46	16,600	5.6	15.2
3	4	45	10,400	5.6	14.8
4	4	47	12,200	8.2	15.6
5	4	45	12,600	6.5	14.8
6	4	47	12,200	6.8	15.2
7	4	45	9,400	5.8	15.6
8	4	45	11,900	7.2	15.6

[a]Packed cell volume reported as percentage of total blood volume.

[b]White blood cell count reported per ml and red blood cell count x 10^6 per ml.

Table 7. Continued

Dog No.	Day Sampled	Blood Parameters			
		PCV[a]	WBC[b]	RBC[b]	Hemoglobin (mg %)
1	6	47	13,300	5.9	15.2
2	6	39	17,000	6.6	18.0
3	6	42	7,000	5.3	15.2
4	6	42	10,800	6.1	14.0
5	6	49	8,300	6.8	15.6
6	6	42	10,700	6.3	14.4
7	6	40	12,300	5.9	13.6
8	6	45	10,600	6.8	15.6
1	9	45	15,700	6.7	17.4
2	9	45	13,600	6.3	15.6
3	9	42	10,100	6.1	15.6
4	9	41	16,100	6.1	14.8
1	12	42	13,600	6.2	18.9
2	12	45	14,900	6.3	16.1
3	12	40	11,200	6.0	16.1
4	12	39	17,100	5.6	14.4

Table 8. Experiment 1: Clinical signs elicited in dogs inoculated intranasally with CHV-270

Dog No.	Day Sampled	Lacri-mation	Conjuncti-vitis	Sneezing	Other Clinical signs
6	2	+[a]	0[b]	+	enlarged tonsils
8	2	+	0	0	
6	4	+	0	0	
8	4	+	0	0	
5	6	0	+	0	
8	6	0	+	0	
6	8	0	+	0	
7	9	0	+	0	
6	12	+	0	0	

[a]Clinical sign present.

[b]Clinical sign absent.

Experiment 2: Pathogenicity of the CHV for the External Genitalia of Male Dogs

Dogs 9, 10 and 11 were inoculated intrapreputially with 5.1×10^5 pfu of CHV-270. The control dog, number 12, received 1 ml of MDCK cell-culture fluid by the same route of inoculation. Blood, pharyngeal, nasal, genital and conjunctival specimens were obtained on days 2, 4, 6, 9, 12, 16, 20 and 28. The results of viral isolations are recorded in Table 9. The virus spread to the nasal cavity, the pharynx, and the conjunctiva, but no viremia could be demonstrated. Virus was isolated from the genital tract for only 6 days, but from the nasal cavity and the conjunctiva for up to 20 days postinoculation.

Table 9. Experiment 2: CHV reisolated from dogs after intrapreputial inoculation of CHV-270

	Days sampled							
Dog No.	2	4	6	9	12	16	20	28
9	G[a]	G	0[b]	C	N	0	0	---[c]
10	G	G	NC	N	0	PC	0	0
11	G	G	NGC	C	PN	NC	NC	0
12 (control)	0	0	0	0	0	0	0	0

[a]G, N and C represent positive CHV isolations from the sheath, nasal cavity and conjunctival sac respectively.

[b]No CHV isolated.

[c]Dog 9 was euthanized for the purpose of a postmortem examination on day 20.

Dog 9 was euthanized on day 20 for the purpose of a postmortem examination. Virus isolation was attempted from penis mucosa, epididymis, testicle, bulbus glandis, prostate, urethra, bladder, kidney, tonsil, lung and trachea. No virus was isolated from any of the above-mentioned tissues. No gross lesions were seen and no microscopic lesions present in sections prepared from the penis mucosa, urethra, epididymis, prostate, kidney, testicle, trachea, heart, liver and lung.

Blood samples for serological tests were obtained on day zero and day 28. All dogs had negative titers to CHV before inoculation and the control dog, number 12, remained negative until day 20 at which time this dog was removed from this experiment so that it could be used in experiment 3. The serological results are recorded in Table 10.

Table 10. Experiment 2: CHV antibody titers in male dogs after intrapreputial inoculation of CHV-270

Dog No.	Days sampled			
	0	14	20	28
9	<2	2	N.D.[a]	---[b]
10	<2	N.D.	N.D.	8
11	<2	N.D.	N.D.	16
12 (control)	<2	N.D.	<2[c]	N.D.

[a]Not done.

[b]Dog number 9 was killed and necropsied on day 16.

[c]Dog number 12 was used in experiment 3 on day 20.

On day 4 the mucosa over the base of the penis and the preputial reflection of dog 9 and dog 10 became reddened and had a roughened granular appearance. The hair at the orifice of the sheath became wet and the dogs licked excessively at the orifice of the sheath. A similar condition appeared in dog 11 on day 5. All of these lesions on the penis regressed in 2 or 3 days. No vesicles were ever observed on the penis mucosa, however small vesicles could have been overlooked as it is difficult to examine the entire penis mucosa of a dog. On day 9 dogs 9 and 11 developed a conjunctivitis which regressed in 2 days.

Experiment 3:
Acute CHV Genital Infection of a Male Dog

Dog 13 was inoculated intrapreputially with 1 ml of fluid containing 7.6×10^5 pfu of CHV-270. Four days after inoculation the dog was euthanized with an overdose of phenobarbital and a postmortem examination was performed. Petechial hemorrhages were seen in the penis mucosa. There were also clear raised areas on the penis mucosa that measured approximately 1 mm in diameter. H and E sections of the penis mucosa revealed that these raised areas were foci of lymphoid infiltration which can be seen in Figure 1. H and E stained sections of testicle, bulbus glandis, epididymis, urethra, lung, liver, kidney, trachea and spleen showed no histological changes.

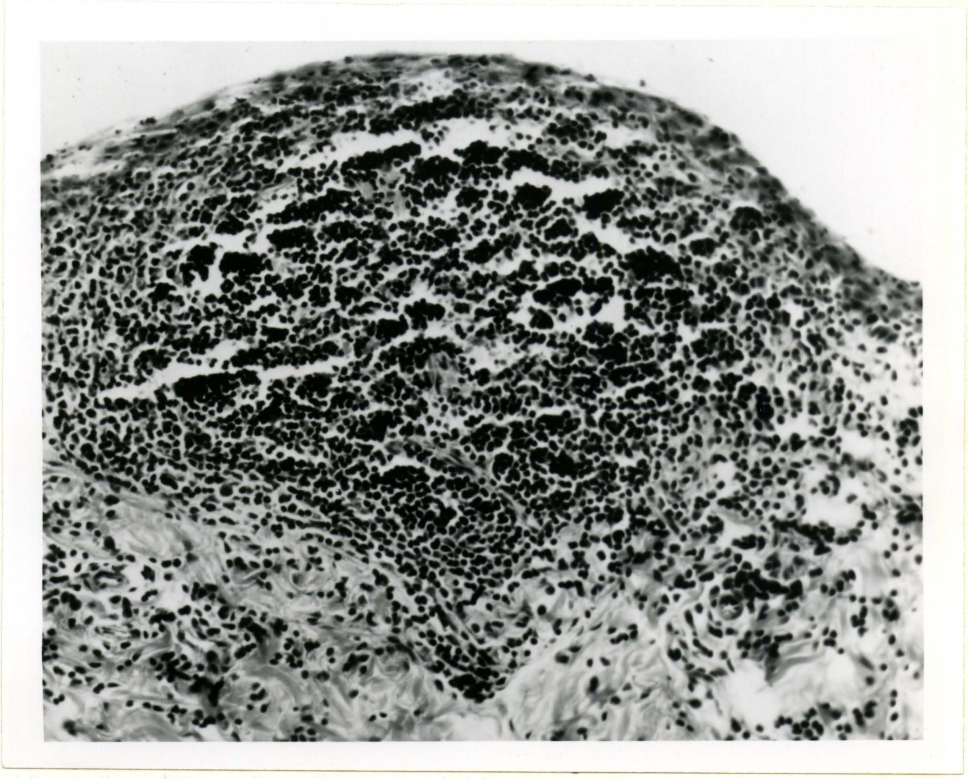

Figure 1. Lymphoid nodule in the penis mucosa of a dog 4 days after intrapreputial inoculation with CHV-270. H and E x 180

Virus isolation was attempted from the above-mentioned tissues as well as bladder, tonsil, nasal swabs, pharyngeal swabs, and genital swabs. CHV was only isolated from the pharyngeal and genital swabs and from the tonsil.

Experiment 4: Pathogenicity of CHV for the Genitalia of Female Dogs

One ml of a viral suspension containing 5.0×10^5 pfu of CHV-270 was inoculated into the vagina of dogs 14, 15, 16 and 17. Dog 18 received 1 ml of MDCK cell-culture also intravaginally. Specimens for virus isolation were obtained on days 2, 4, 6, 9, 12, 16 and 20 and blood for serologic tests was collected on days zero and 28. As in previous experiments the infected dogs developed neutralizing antibodies to CHV-270 while the control remained negative (Table 12). It can be seen from Table 11 that the virus did replicate in the vagina as CHV was isolated from the vagina for 6 days postinoculation. However, it did not spread much as the virus was only isolated from one site other than the genital tract, that site being the conjunctiva of dog 14.

The lesions that were produced in the vagina of the CHV-270 infected dogs were quite dramatic (Table 13). Two days after inoculation all dogs developed a severe vaginitis and dog 14 had a slight purulent vaginal discharge. Several 1 mm glistening white raised areas were present in the vaginal

Table 11. Experiment 4: CHV reisolated from dogs after intravaginal inoculation of CHV-270

Dog No.	Days sampled						
	2	4	6	9	12	16	20
14	G[a]	G	GC	C	0[b]	0	0
15	G	G	G	0	0	0	0
16	G	G	G	0	0	0	0
17	G	G	G	0	0	0	0
18 (control)	0	0	0	0	0	0	0

[a]G and C represent positive CHV isolations from the vagina and conjunctival sac, respectively.

[b]No CHV isolated.

Table 12. Experiment 4: CHV antibody titers in dogs after intravaginal inoculation of CHV-270

Dog No.	Days sampled	
	0	28
14	<2	32
15	<2	8
16	<2	8
17	<2	16
18 (control)	<2	<2

Table 13. Experiment 4: Clinical signs elicited in dogs inoculated intravaginally with CHV-270

Dog No.	Day	Vaginal lesions			Conjunctivitis
		Vaginitis	Lymphoid nodules	Petechial or submucosal hemorrhages	
14	2-5	+[a]	+	+	0[b]
15	2-5	+	+	+	0
16	2-5	+	+	+	0
17	2-5	+	+	+	0
17	5	0	0	0	0
14	6	+	0	0	0
15	6	0	0	+	0
16	6	0	0	+	0
17	6	+	0	+	0
16	7	0	0	0	+
14	9	0	0	0	+
16	9	0	0	0	+
14	12	0	0	0	+
16	12	0	0	0	+
14	16	0	0	0	+
16	16	0	0	0	+

[a]Clinical sign present.

[b]Clinical sign absent.

mucosa of each dog. These white areas appeared as vesicles but examination of H and E sections of these affected areas of vaginal mucosa revealed that they were lymphoid nodules (See Figure 4). The vaginal mucosa also contained many petechial and submucosal hemorrhages. The majority of the lesions were on the dorsal and lateral aspects of the vestibule. A dry crusty lesion also appeared in infected dogs at the dorsal commissure of the vulva right at the mucocutaneous junction. On day 5, dog 17 had a yellowish vaginal exudate which lasted for 2 days. A staphylococcus and a streptococcus were isolated from this exudate, but CHV was also isolated from the vagina of this dog on day 6.

The vaginal lesions were regressing by day 6 and were completely gone by day 7.

The only other symptom seen in these dogs was conjunctivitis that developed in dogs 14 and 16 on day 7. This condition lasted for about 10 days.

Experiment 5: Protection Provided by CHV-33 when Administered by Different Routes of Inoculation and Challenged Intravaginally with CHV-270

This experiment was designed to test the virulence and immunogenicity of CHV-33. Dogs 19, 20, 21 and 22 were controls and did not receive anything on day 0. Dogs 23, 24, 25 and 26 were inoculated intravaginally with 1 ml of CHV-33 containing 5.0×10^5 pfu of virus. Dogs 27, 28, 33 and 34 were inoculated intranasally with the same amount of CHV-33 and dogs 29, 30, 31 and 32 were inoculated subcutaneously, also with 5.0×10^5 pfu of CHV-33. Each group of 4 dogs was maintained in isolation cages (2 dogs per cage) so that no contact spread could occur between groups.

All sixteen dogs were challenged intravaginally with 5.1×10^5 pfu of CHV-270 on day 28. The intravaginal route was chosen for challenging because the vagina was the only site which responded in a recognizable manner to CHV 100 percent of the time. Each dog was sampled on day 2, 4, 6, 9, 12, 16 and 20 after both the CHV-33 inoculation and the CHV-270 challenge.

The virus isolations, serological results and clinical signs from this experiment are recorded in Tables 14, 15 and 16, respectively. When inoculated intranasally or subcutaneously CHV-33 caused an antibody response but when inoculated intravaginally no antibody response occurred.

Table 14. Experiment 5: CHV reisolated from dogs inoculated by various routes with CHV-33 and then challenged intravaginally with CHV-270

Dog No.	Route of CHV-33 Inoculation	Days sampled							CHV-270 challenge on day 28							
		2	4	6	9	12	16	20		30	32	34	37	40	44	48
19	Control	0	0	0	0	0	0	0		G[a]	G	PNGC	C	N	N	0
20		0	0	0	0	0	0	0		G	GC	C	C	C	C	0
21		0	0	0	0	0	0	0		G	GC	PGC	C	NC	N	0
22		0	0	0	0	0	0	0		C	C	NG	0	PN	N	0
23	Intravaginal	0	G	G	0	0	0	0		C	0	0	0	0	0	0
24		G	G	G	0	0	0	0		G	G	0	0	0	0	0
25		0	G	G	0	0	0	0		C	0	0	0	0	0	0
26		0	0	0	0	0	0	0		G	G	0	0	0	0	0
27	Intranasal	PN	N	N	0	0	0	0		G	G	0	0	0	0	---
28		N	N	N	P	P	0	0		G	G	0	0	0	0	0
33		PN	G	0	0	0	0	0		0	0	0	0	0	0	0
34		NC	PNC	PN	N	0	0	0		G	0	0	0	0	0	0

[a] G, N, P and C represent positive CHV isolations from the genital, nasal cavity, pharynx and conjunctival sac, respectively.

Table 14. Continued

Dog No.	Route of CHV-33 Inoculation	Days sampled							CHV-270 challenge on day 28							
		2	4	6	9	12	16	20		30	32	34	37	40	44	48
29	Subcutaneous	PN	N	0	0	0	0	0		G	C	G	0	0	0	0
30		N	N	0	N	0	0	0		0	0	G	0	0	0	0
31		NC	PNC	PC	P	0	0	0		G	G	0	0	0	0	0
32		0	0	0	0	0	0	0		G	G	G	0	0	0	0

Table 15. Experiment 5: CHV antibody titers of dogs inoculated by various routes and then challenged intravaginally with CHV-270 on day 28

Dog No.	Route of Inoculation	Antibody titers		
		Day 0	Day 28	Day 56
19	Control	<2	<2	64
20		<2	<2	32
21		<2	<2	16
22		<2	<2	16
23	Intravaginal	<2	<2	16
24		<2	<2	16
25		<2	<2	16
26		<2	<2	32
27	Intranasal	<2	4	N.D.[a]
28		<2	16	N.D.
33		<2	16	N.D.
34		<2	16	N.D.
29	Subcutaneous	<2	16	N.D.
30		<2	16	N.D.
31		<2	16	N.D.
32		<2	16	N.D.

[a]Not done.

Table 16. Experiment 5: Clinical signs elicited in dogs inoculated by various routes with CHV-33 and then challenged intravaginally with CHV-270 on day 28

Dog No.	Day	Vaginal lesions: Vaginitis	Vaginal lesions: Lymphoid nodules	Vaginal lesions: Petechial or submucosal hemorrhages	Other clinial signs
25	2	+[a]	0[b]	+	
27	2	0	0	0	lacrimation and rhinorrhea
23	3	+	0	+	
24	3	+	0	+	
25	3	+	0	+	
27	3	0	0	0	pulmonary congestion, rales
31	3	0	0	0	conjunctivitis
34	3	0	0	0	conjunctivitis
23	4	+	0	+	
24	4	+	+	+	
25	4	+	0	+	
26	4	+	+	0	
31	4	0	0	0	severe conjunctivitis
33	4	+	0	0	
34	4	0	0	0	severe conjunctivitis

[a]Clinical sign present.

[b]Clinical sign absent.

Table 16. Continued

Dog No.	Day	Vaginal lesions: Vaginitis	Vaginal lesions: Lymphoid nodules	Vaginal lesions: Petechial or submucosal hemorrhages	Other Clinical signs
23	5	+	+	+	
24	5	+	+	+	
25	5	+	+	0	
26	5	+	0	+	
31	5	0	0	0	conjunctivitis
34	5	0	0	0	conjunctivitis
23	6	+	+	+	
24	6	+	+	+	
25	6	+	+	+	
25	7	+	0	+	
19	30	+	0	+	vaginal discharge
20	30	+	+	+	
21	30	+	0	+	
22	30	+	+	0	
30	30	+	+	+	
31	30	+	0	+	
19	31	+	0	0	vaginal discharge
20	31	+	+	0	
21	31	+	+	0	
22	31	+	0	+	
23	31	+	+	0	

Table 16. Continued

Dog No.	Day	Vaginal lesions: Vaginitis	Vaginal lesions: Lymphoid nodules	Vaginal lesions: Petechial or submucosal hemorrhages	Other clinical signs
19	32	+	0	0	
20	32	+	0	0	
21	32	+	+	0	
22	32	+	+	0	
23	32	+	0	0	
24					
25	32	Slight vaginal inflammation - no vesicles			
26					
19	33	+	0	0	
20	33	+	0	0	
21	33	+	+	0	
22	33	+	+	0	
23					
24					
25	33	Slight vaginal inflammation - no vesicles			
26					
22	34	+	+	0	
29	34	+	+	0	vaginal discharge
30	34	+	+	0	
32	34	+	+	0	
29	37	+	+	0	
32	37	+	+	0	

This is in contrast to the antibody response observed after intravaginal inoculation of CHV-270 in experiment 4 and after intravaginal challenge of the control dog in experiment 5. The spread of virus after challenge with CHV-270 was considerably more than was noted in experiment 4 with intravaginal inoculation with CHV-270. Intravaginal, intranasal and subcutaneous inoculation with CHV-33 seems to provide some protection at least as far as virus spread was concerned. Only one of the 12 dogs inoculated by these routes yielded virus from a site other than the vagina.

In those dogs inoculated intravaginally with CHV-33, vaginal lesions like those described in experiment 4 developed (See Figures 2, 3, 4 and 5). After intravaginal challenge with CHV-270, these dogs again developed vaginitis but the lesions were much less severe and only one dog developed lymphoid nodules (only 2 nodules noted).

Virus was isolated from 2 or more sites in 3 of the 4 dogs inoculated subcutaneously with CHV-33. This would indicate a spread of the virus by some other means than physical contact. In 2 of these 4 dogs a severe conjunctivitis developed. On day 4 the conjunctivitis was so severe that the eyes were swollen shut. The conjunctivas of these dogs was bright red due to the inflammation and the dogs were very sensitive to any manipulation of the eyelids. CHV was isolated from the eyes of both of these dogs during the time they had the conjunctivitis.

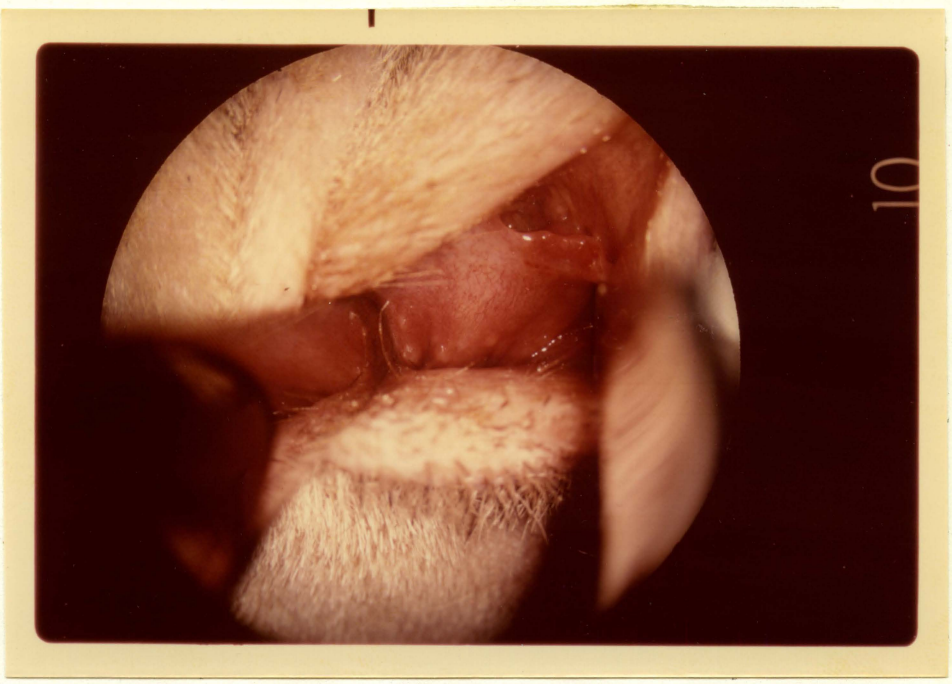

Figure 2. Vaginal mucosa of dog 30, 6 days after intravaginal challenge with CHV-270. Note the lymphoid nodules

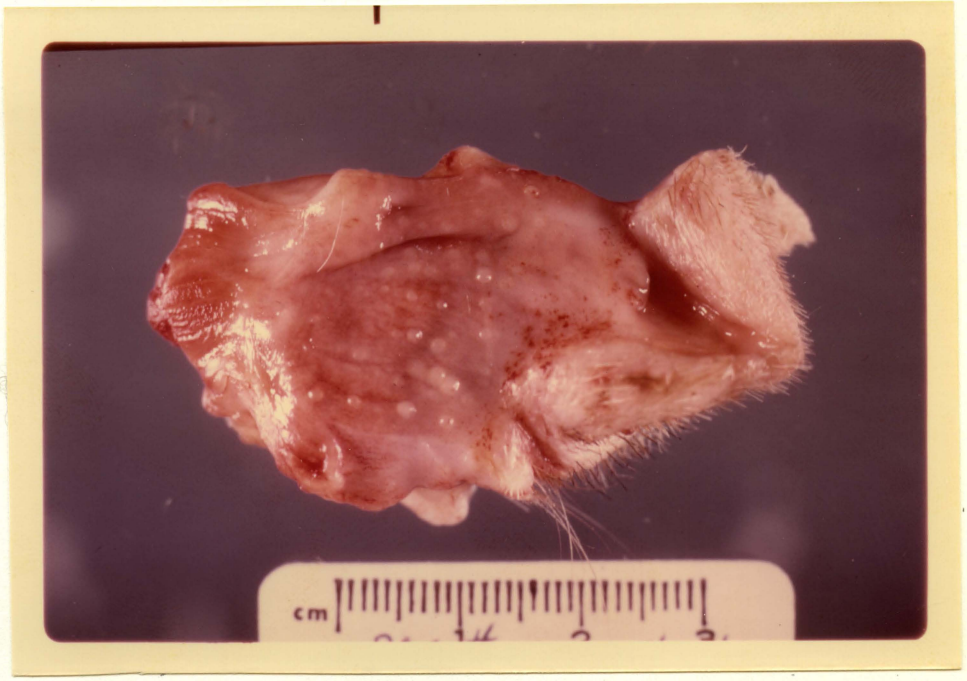

Figure 3. Lymphoid nodules and petechial hemorrhages in the vaginal mucosa of dog 30, 6 days after intravaginal challenge with CHV-270

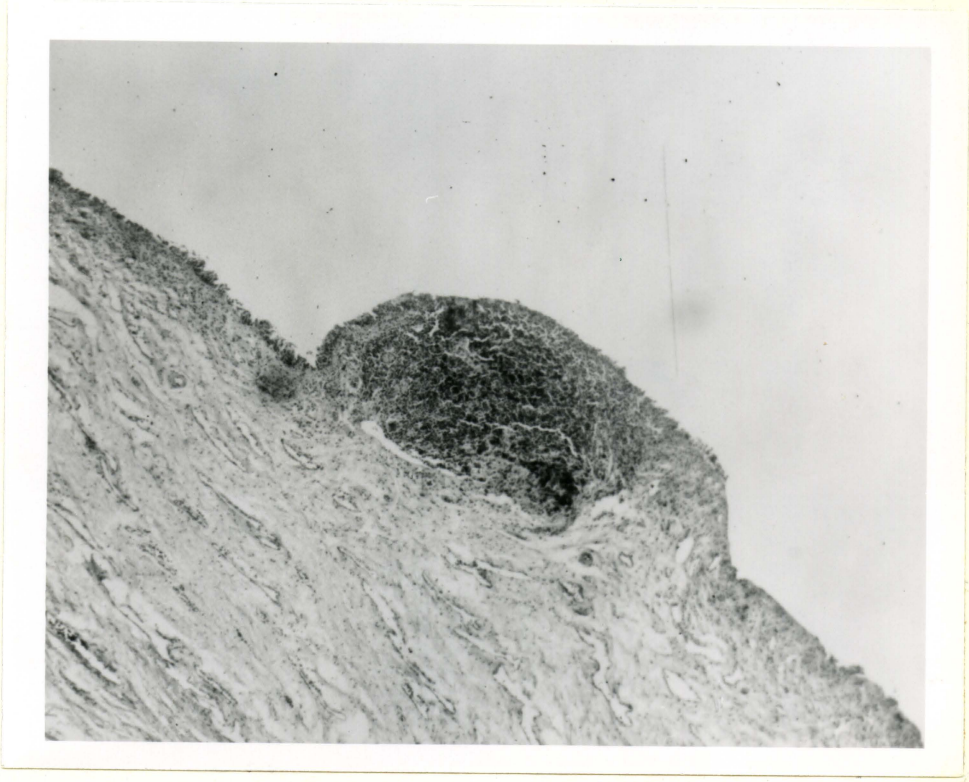

Figure 4. Lymphoid nodule in the vaginal mucosa of dog 30, 6 days after intravaginal challenge with CHV-270. H and E x 72

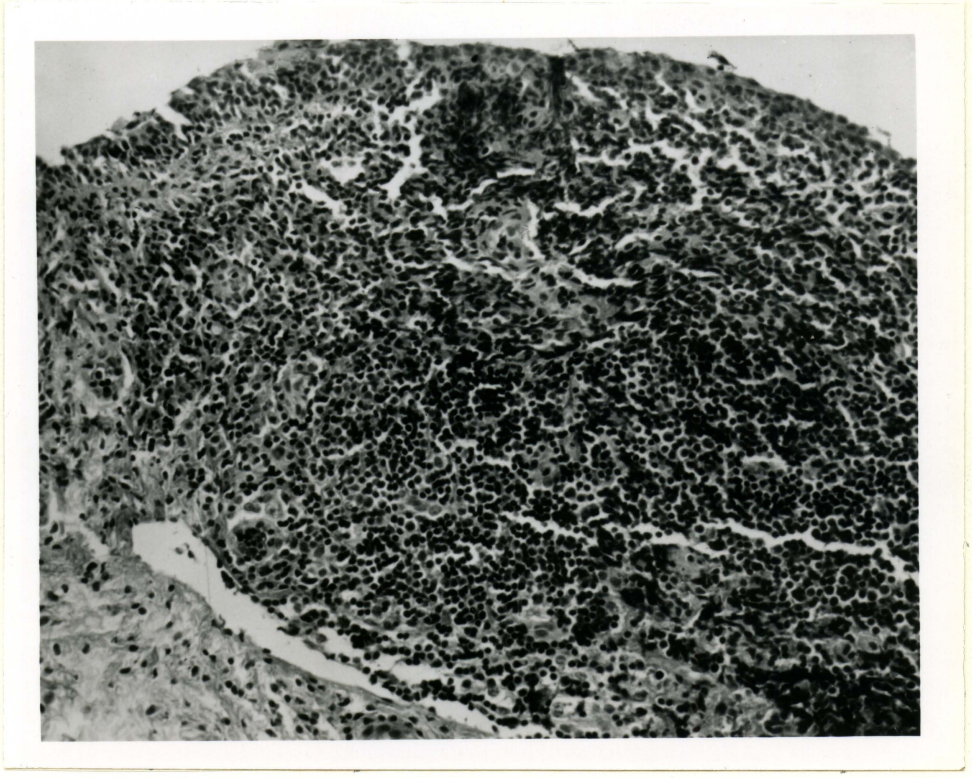

Figure 5. Lymphoid nodule in the vaginal mucosa of dog 30, 6 days after intravaginal challenge with CHV-270. H and E x 180

Of the 12 dogs inoculated with CHV-33, 5 dogs (27, 28, 31, 33 and 34) failed to show clinical signs while only dog 33 failed to yield virus when challenged with CHV-270.

Experiment 6: The Effect of Environmental Temperature on the Pathogenicity of CHV on Dogs Inoculated Intranasally

Three 16-week-old dogs were placed in an environmental chamber[1] in which the temperature was continually varied from 22 C (72 F) during the day (12 hours) to 0 C (32 F) during the night (12 hours). After a 5-day adjustment period, these dogs were inoculated intranasally with 0.5 ml of MDCK cell-culture fluid per naris. These dogs were observed twice a day for any clinical signs. All 3 dogs remained normal and they did not develop any CHV antibody titer by day 28.

Another group of three 16-week-old dogs was placed in this environmental chamber and again the temperature was varied from 22 C (72 F) to 0 C (32 F) on 12 hour cycles. After a 5-day adjustment period, these 3 dogs were inoculated intranasally with 0.5 ml per naris of a viral suspension containing 5.1×10^5 pfu of CHV-270.

As can be seen from Table 17, dog 38 had a titer of 8 on day zero. On day 28, this dog had a titer of 64 while dogs 39 and 40 had titers of 16. This preinoculation titer

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[1]CER-810 Controlled Environment Room, Sherer-Gillett, Marshall, Michigan.

in dog 38 did not prevent the CHV infection because as can be seen from Table 18, virus was isolated from this dog for 6 days. However, the symptoms were not as severe in this dog as they were in dogs 39 and 40. On day 2 postinoculation dog 39 was sneezing and had a slight rhinorrhea. By the next day dogs 39 and 40 were sneezing violently and both had rhinorrhea. On day 4 all dogs were sneezing and when swabbing the nose a blood-tinged pink fluid was noted on the cotton swabs. Dog 38 only sneezed for approximately one day but dogs 39 and 40 sneezed until day 8. On day 5 both of these dogs developed a conjunctivitis that also lasted until the 8th day postinoculation.

Table 17. Experiment 6: Antibody titers after intranasal inoculation of CHV-270 in dogs maintained at varying environmental temperatures

Dog No.	Days sampled	
	0	28
35 (control)	<2	<2
36 (control)	<2	<2
37 (control)	<2	<2
38	8	64
39	<2	16
40	<2	16

Table 18. Experiment 6: CHV reisolated from dogs inoculated intranasally and maintained at varying environmental temperatures

Dog No.	Days sampled				
	2	4	6	9	12
38	NP[a]	NP	N	0[b]	0
39	NP	NP	NC	0	0
40	NPC	NP	NC	C	0

[a]N, P and C represent positive CHV isolations from the nasal cavity, pharynx and conjunctival sac, respectively.

[b]No CHV isolated.

Experiment 7: Intranasal and Intraconjunctival Inoculation of CHV in Dogs Maintained at Normal and Varying Environmental Temperatures

Eight 10-week-old dogs were divided into 2 groups of 4 dogs each. One group was placed in an environmental chamber and the other group was placed in plastic isolation cages. The temperature in the environmental chamber was varied from 22 C (72 F) to 0 C (32 F) at 12 hour intervals. After a 5-day adjustment period 2 dogs maintained in the varying temperature (dogs 41 and 42) and 2 dogs (45 and 46) maintained at normal room temperature 22 C (72 F) were inoculated by injecting 0.25 ml of a CHV-270 suspension containing 1.5×10^6 pfu/ml into the conjunctival sac of each eye. The other 2 dogs in each group, dogs 43, 44, 47 and 48, were inoculated intranasally with 1.0 ml of suspension of CHV-270 containing 7.5×10^5 pfu/ml.

Blood samples were obtained before inoculation and at 7 day intervals for serological tests. Pharyngeal, nasal, genital, and conjunctival samples were obtained on days 2, 4, 6, 9, 12, 16 and 20 for viral isolation.

As can be seen in Table 20, all dogs had preinoculation antibody titers to CHV. It was decided to proceed with the experiment using these dogs because the positive preinoculation titers would add an interesting dimension to this experiment, that is the effects that circulating CHV neutralizing antibodies would have on subsequent reinfection.

By comparing the clinical signs in Table 21 and viral isolations in Table 19, with the CHV antibody titers in Table 20, it is apparent that a high titer protected dog 41 from a severe disease but it did not prevent this dog from reinfection as CHV was isolated for 9 days postinoculation. Low CHV antibody titers (4-16) seen in all the other dogs neither protected the dogs from severe clinical signs nor from infection.

The route of inoculation did not seem to have any effect on the clinical symptoms as the infections spread from the nasal cavity to the eye and vice versa.

The environmental temperature at which these dogs were maintained had a profound effect on the severity of the clinical symptoms that developed. All the dogs maintained at a constant normal room temperature developed mild conjunctivitis and rhinorrhea similar to what was seen in experiment 1. However, the clinical symptoms seen in the dogs maintained in the varying temperatures were much more severe with the exception of dog 41 which had the comparatively high preinoculation CHV antibody titer. The sneezing which was seen in experiment 6 was again seen in dogs 42, 43 and 44. More severe signs in the 3 dogs in experiment 7 was possibly due to a difference in age. The dogs in experiment 7 were 10 weeks old at the onset of the experiment which may have accounted for the

Table 19. Experiment 7: CHV reisolated from dogs maintained at different environmental temperatures and inoculated with CHV-270

Dog No.	Environmental Temperature	Days sampled						
		2	4	6	9	12	16	20
41	varied[a]	0[b]	C[c]	C	C	0	0	0
42	varied	C	CNP	P	CN	(died)		
43	varied	N	NP	CNP	CN	(died)		
44	varied	N	CNP	P	0	0	0	0
45	22 C	0	CNP	CN	0	0	0	0
46	22 C	CP	CNP	N	0	0	0	0
47	22 C	NP	NP	NP	NP	0	0	0
48	22 C	NP	CNP	N	0	0	0	0

[a]Temperature was varied on a 12 hour cycle between 22 C and 0 C.

[b]No CHV isolated.

[c]C, N and P represent positive CHV isolations from the conjunctival sac, nasal cavity and pharynx, respectively.

Table 20. Experiment 7: CHV antibody titers of dogs maintained at different environmental temperatures and inoculated with CHV-270

Dog No.	Environmental temperature	Days sampled		
		0	7	28
41	varied	256		256
42[a]	varied	4	8	
43[a]	varied[b]	16	16	
44	varied	4		512
45	22 C	16		64
46	22 C	16		64
47	22 C	4		256
48	22 C	16		512

[a]Dogs 42 and 43 died on day 9 and 11 postinoculation, respectively.

[b]Temperature was varied on a 12 hour cycle between 22 C and 0 C.

Table 21. Experiment 7: Clinical signs elicited in dogs maintained at different environmental temperatures and inoculated with CHV-270

Dog No.	Day	Conjunctivitis	Rhinorrhea	Sneezing
42	2	±[a]	0[b]	0
43	2	±	+	0
44	2	±	+	0
41	4	+	0	0
42	4	++[c]	+	+
43	4	++	+	+
44	4	++	+	+
41	6	+	0	0
42	6	++	++	+
43	6	++	++	+
44	6	+	0	+
47	6	±	±	0
48	6	±	±	0
41	8	±	0	0
42[d]	8	++	++	+
43	8	++	++	+
44	8	+	+	0
45	8	±	±	0

[a]Indicates the condition was present but to a lesser degree than +.

[b]Clinical sign absent.

[c]Indicates a greater severity of the lesion than +.

[d]Dogs 42 and 43 died on days 9 and 11, respectively.

Table 21. Continued

Dog No.	Day	Conjunctivitis	Rhinorrhea	Sneezing
46	8	±	±	0
47	8	±	±	0
48	8	±	±	0
43[d]	11	++	++	+
44	11	+	+	+
44	12	±	±	0

reduced resistance in comparison to the dogs in experiment 6, which were 16 weeks old. All 4 dogs in experiment 7 that were maintained at varying environmental temperatures were able to maintain a normal body temperature during the preinoculation period. However, on the 2nd day postinoculation the temperature of all 4 of these dogs dropped (See Table 22).

Clinical symptoms were evident in dogs 42, 43 and 44 by day 2 postinoculation. The clinical symptoms in dogs 42 and 43 increased in severity until day 4 when both dogs had a severe conjunctivitis and rhinitis. The conjunctivitis was characterized by a severely injected sclera and conjunctiva with copious amounts of purulent exudate draining and encrusting around the eyes. The rhinitis was characterized by a mucopurulent rhinorrhea and sneezing. Figure 6 is a photograph of dog 42 on day 6 postinoculation. Dog 44 also developed severe conjunctivitis but by day 6 postinoculation this dog was showing some improvement while the conditions of dogs 42 and 43 continued to deteriorate. Both of these dogs lost body weight and condition and their hair coats became rough, dry and scaly. The deteriorating condition of dogs 42 and 43 climaxed in death on day 9 and day 11, respectively. Figure 7 is a photograph of dog 42 shortly after death.

At the time of death the small intestines of both dogs

Table 22. Experiment 7: Rectal temperatures of dogs maintained at different environmental temperatures and inoculated with CHV-270

Dog No.	Environmental temperature	Average pre-inoculation temperature	Days postinoculation 1	2	4	6	9
41	varied	101.3	101.2	100.0	99.4	99.6	99.4
42	varied	101.2	101.2	99.6	98.8	98.0	98.2
43	varied	101.9	101.4	99.8	100.0	99.0	(died)
44	varied	101.4	101.2	100.2	99.2	100.0	99.0
45	22 C	101.5	101.4	101.0	101.2	101.4	101.0
46	22 C	101.5	100.8	101.2	101.5	102.0	102.0
47	22 C	101.3	103.0	101.8	101.6	102.8	102.4
48	22 C	101.2	101.6	102.8	104.6	101.6	101.0

Figure 6. Dog 42, maintained at varying environmental temperatures, 6 days after intraocular inoculation of CHV-270. Note conjunctivitis and ocular discharge

Figure 7. Dog 42 on day 9 postinoculation, shortly after death. Note the exudate encrusted around the eyes and nose

were congested and there were microscopic hemorrhages at the tips of the villi. Both dogs had foci of pulmonary hemorrhage (Figure 8) but the overall histopathological appearance of the lungs was one of purulent bronchopneumonia and hyperemia. Histopathological examination of the conjunctiva, brain and turbinates revealed a purulent necrotizing conjunctivitis, rare glial nodules in the medulla, and a purulent necrotizing rhinitis, respectively. Figures 9, 10 and 11 depict the above-mentioned lesions.

Bordetella bronchiseptica was isolated from the lungs of both dogs that died.

Figure 8. Lung from dog 42, 9 days after intraocular inoculation of CHV-270. Note foci of pulmonary hemorrhages

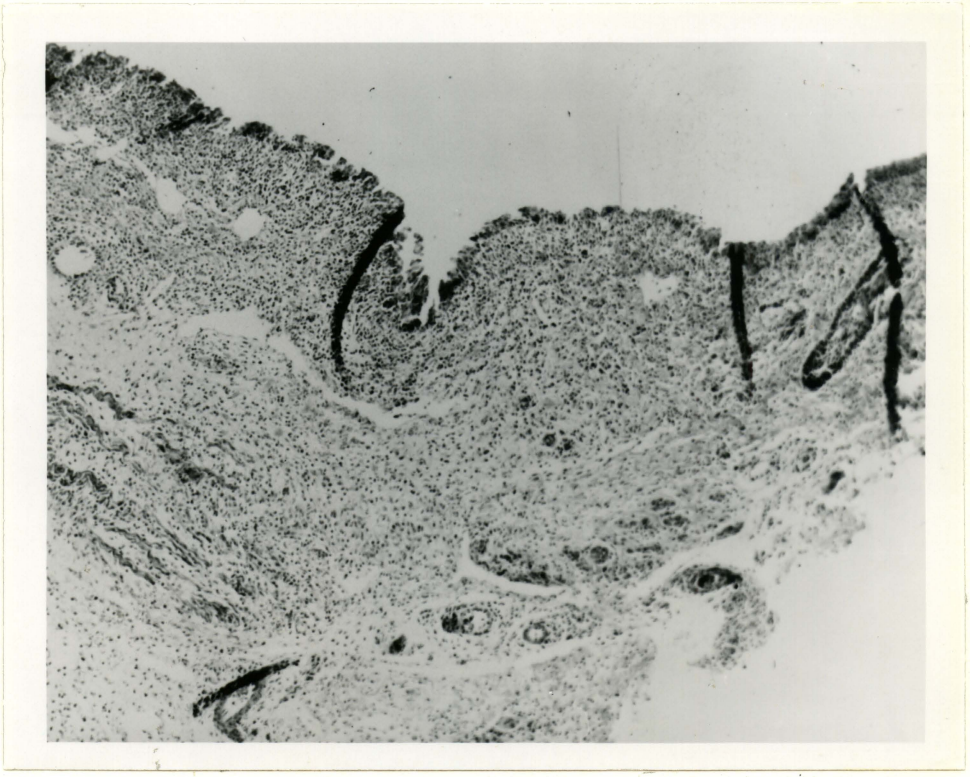

Figure 9. Conjunctiva from dog 43. Exudative necrotizing conjunctivitis. Dog 43 was inoculated intranasally with CHV-270 and maintained at varying environmental temperatures. H and E x 72

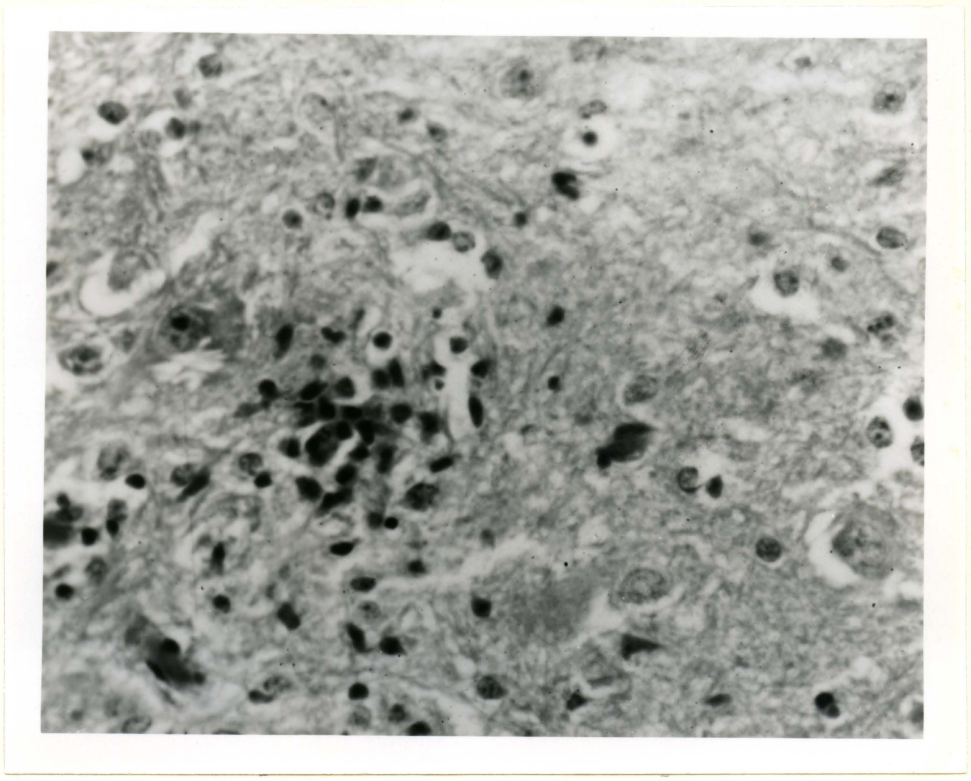

Figure 10. Medulla from dog 43 inoculated intranasally with CHV-270. Note glial nodule. H and E x 450

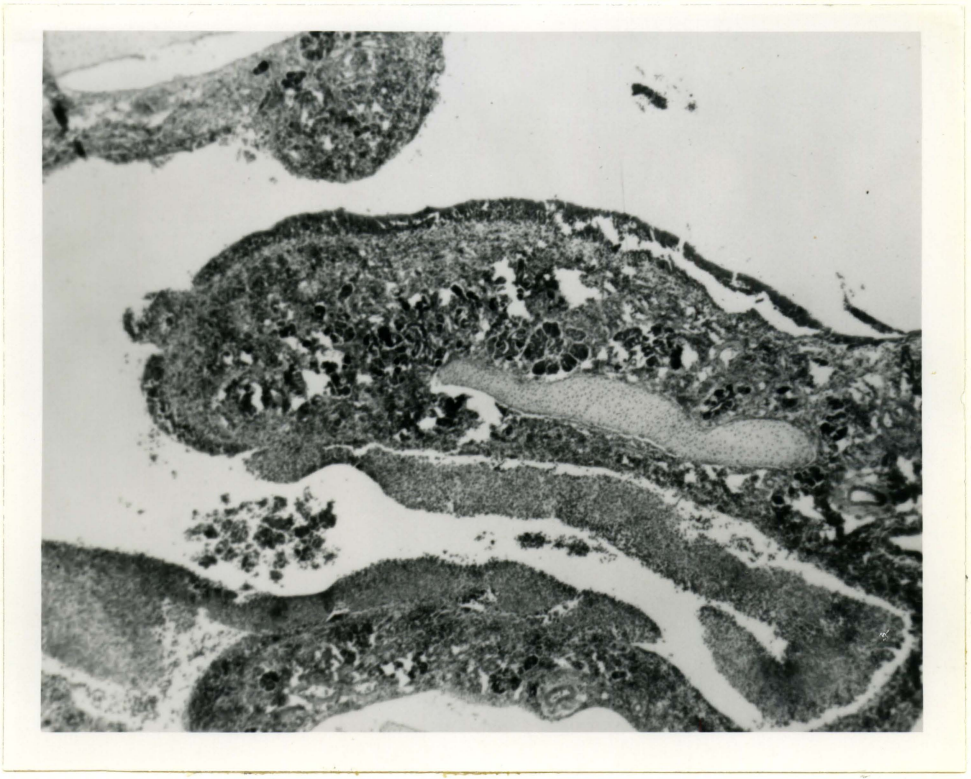

Figure 11. Nasal turbinates from dog 43 inoculated with CHV-270. Note purulent necrotizing rhinitis. H and E x 72

Experiment 8: The Effects of *Bordetella bronchiseptica* on CHV Infections of Dogs Maintained at Varying Environmental Temperatures

This experiment was designed to determine the effect of *B. bronchiseptica* in CHV infection. Varying environmental temperature was used, as in experiments 6 and 7, as a factor to elicit recognizable symptoms. Four 14-week-old German Shepherd crossbred dogs were used for this experiment. All dogs were seronegative for CHV on day zero. After a 5-day adjustment period dogs 51 and 52 were intranasally inoculated with 1 ml of a 1 to 2 dilution of a 36 hour *B. bronchiseptica* culture grown in tryptose-phosphate broth. Dogs 49 and 50 received 1 ml intranasally of uninoculated tryptose-phosphate broth at the same time. All four dogs remained symptomless for 7 days at which time *B. bronchiseptica* was reisolated from the nasal cavity of dogs 51 and 52 and from the pharynx of dog 52. Dogs 49 and 50 remained negative for *B. bronchiseptica*. On day 7 all dogs were intranasally inoculated with 1 ml of a suspension of CHV containing 5.1×10^5 pfu.

All dogs were observed for 2 weeks after viral inoculation for the appearance of clinical signs which were delayed until day 12 (5 days after viral inoculation). Dogs under similar conditions in previous experiments developed signs by day 2 or 3. This delay of symptoms was attributed to a malfunction in the chamber which allowed the temperature to

rise to 32 C (90 F) during the 0 C (32 F) cycle on day 9. Clinical symptoms characterized by conjunctivitis, rhinorrhea, and sneezing commenced on day 12 and lasted until day 17. The severity of the clinical signs was essentially the same as described in experiment 6. The severity of clinical signs between dogs was similar throughout the course of the 5-day period.

On day 15 dog 50 became positive for B. bronchiseptica, by bacterial isolation. Dog 49 remained negative for B. bronchiseptica while dogs 51 and 52 remained positive throughout the experiment.

Table 23. Experiment 8: CHV antibody titers in dogs after intranasal inoculation of CHV-270

	Days sampled	
Dog No.	0	21
49	<2	64
50	<2	64
51[a]	<2	16
52[a]	<2	16

[a]Dog 51 and dog 52 were inoculated with B. bronchiseptica intranasally on day zero.

DISCUSSION

CHV as a Pathogen of the Respiratory Tract

The CHV has been reported to cause a relatively mild or inapparent upper respiratory infection in non-neonatal dogs (2, 116). However, the CHV isolated by Karpas et al. (66) was reported to cause canine tracheobronchitis under natural as well as experimental conditions. It is impossible to draw conclusions from these conflicting reports as the two groups of workers were using different strains of CHV. Although cross SVN tests between CHV-270 and CHV F-205 conducted in this study indicate that these two strains are serologically similar they may differ biochemically, which could be responsible for a difference in pathogenicity as seen in the case of H. hominis type 1 and type 2 (10, 80). This is an acceptable theory as to why there are conflicting reports of pathogenicity using these two strains of virus although it does not seem to be the right theory on the basis of the experiments performed in this study.

The results of intranasal inoculation of dogs maintained at normal environmental temperatures with CHV in this study would support the findings of Appel et al. (2), their findings being that CHV causes a respiratory infection which is mild or inapparent in non-neonatal dogs. Throughout the experiments described previously, dogs inoculated intranasally with CHV-270 failed to show the symptoms of canine

tracheobronchitis described by Karpas et al. (66).

Karpas et al. (67) isolated CHV from both clinically ill and normal dogs in a group of 75 dogs. CHV was isolated from 7 of 45 clinically normal dogs and from only 5 of 30 dogs with clinical signs of respiratory disease described as canine tracheobronchitis. With these kinds of results it would seem questionable that CHV was the cause of this disease. It is possible that the CHV infection in this group of dogs was coincidental, or perhaps only one of several contributing factors to the tracheobronchitis. Other workers (12) have isolated CHV from dogs with respiratory disease but little significance has been placed on the findings because of the low incidence of recovery and the recovery of other agents from these dogs. Karpas et al. (67) were able to produce the typical paroxysmal cough associated with canine tracheobronchitis in only three of fifteen dogs inoculated intranasally with CHV.

In summary, the role of the CHV as a respiratory pathogen of dogs maintained at normal environmental conditions, as defined in this study, completely supports the findings of Appel et al. (2). Both CHV F-205 and CHV-270 are only mildly pathogenic for the canine respiratory tract under normal circumstances. No conclusions can be drawn between the pathogenicity of these two isolates and the British isolate of Wright and Cornwell (116) which causes no respiratory symptoms. As explained previously, the difference

in pathogenicity may actually be due to subtle differences in the virus isolates rather than in host susceptibility, host microbial flora, or acuity of the workers' observations.

It is apparent from this study that the clinical respiratory disease can be increased dramatically when dogs are maintained at cyclic environmental temperatures between zero and 22 C (32-72 F) as in experiments 6, 7 and 8. Previous work by Aurelian (7) has shown that *in vitro* yields of CHV were dependent on the temperature of cultivation. Temperatures above and below 37 C resulted in decreased yield of CHV. Carmichael *et al.* (26) theorized that increasing body temperature may be the cause of decreased susceptibility of the dog to CHV with increasing age. Their experiments showed that increasing the environmental temperature in which the CHV infected neonatal pups were maintained would decrease the pathogenicity. These same workers maintained 4 to 8-week-old dogs in a hypothermic state for 45-48 hours by tranquilization and found that these dogs had significantly greater amounts of viral antigen in their tissues, as demonstrated by the fluorescent-antibody technique, and by the greater amount of recoverable CHV from the tissues in comparison to control dogs infected with CHV and maintained at normal temperatures. They were also able to demonstrate histological lesions in the dogs in a hypothermic state while control dogs lacked such lesions.

It is hypothesized that in experiments 6, 7, and 8 the environmental temperature was lowered sufficiently to cool the respiratory epithelium to a temperature optimal for CHV replication. This could explain the increased pathogenicity of CHV respiratory infection in the dogs maintained at lower environmental temperatures. In experiment 7 there was an added benefit of lowering the environmental temperature which was the resultant reduction in body temperature of the 4 dogs. It can be postulated that this lowered body temperature also added to the increased severity of the CHV infections in this experiment which resulted in the death of two of the dogs. The lowered body temperature seen in the 4 dogs was not due entirely to the lowered environmental temperature because there was no reduction in body temperature in these dogs during the 5-day adjustment period in which environmental temperature was lowered as it was after inoculation. It appears that possibly a combination of the lowered environmental temperature and the CHV infection caused the lowering of the body temperature. The reason that the 4 dogs in experiment 7 had a reduced body temperature while dogs in experiments 6 and 8 had normal body temperatures during these experiments is probably due to age and general body conditioning. Dogs in experiment 7 were 4 to 6 weeks younger and appeared to have less body fat than dogs in experiments 6 and 8. The

fact that the dogs in experiment 7 came from different sources than the dogs in experiments 6 and 8 caused concern because of the probable difference in resident microflora which might also account for variation in the severity of the disease. The isolation of B. bronchiseptica from the lungs of both dogs that died in experiment 7 initiated the interest in the auxiliary role that B. bronchiseptica might have in a CHV infection. Appel et al. (4) stated that B. bronchiseptica were the most common bacteria isolated from dogs with respiratory disease in their study and that bacteria are very important secondary invaders in canine respiratory infections. Experiment 8 failed to show that concurrent B. bronchiseptica and CHV infections caused an increase in respiratory symptoms over those caused by CHV alone. However, it is possible that B. bronchiseptica was important as a secondary bacterial invader which contributed to the demise of dogs 42 and 43 in experiment 7.

CHV as a Pathogen of the Genital Tract

Previous to the report by Poste and King (90) of the isolation of CHV from the genital tract of dogs experiencing infertility, abortion, and stillbirths, little attention had been paid to CHV as a genital tract pathogen of dogs. It had been reported that CHV F-205 caused a mild inflammation of the vaginal mucosa after intravaginal inoculation (2). In this study we were able to show that CHV-270 was pathogenic for the genital tract of both male and female dogs.

Poste and King described the early lesions they saw as vesicular in nature, later becoming "pock-like." The lesions seen in this study also looked vesicular, having glistening white surfaces. However, these lesions, which on histological examination were found to be lymphoid nodules rather than vesicles, never became "pock-like."

Other differences between the lesions reported by Poste and King and those observed in this study were the duration of the visible lesions, the length of the incubation period, and the incidence of genital infection after intravaginal inoculation of CHV. Poste and King (90) reported that vesicles last for 14 to 18 days in naturally-infected dogs while the vaginal lesions in this study lasted only 4 to 5 days. Also in this study, bitches previously unexposed to CHV developed vaginal lesions 2 days postinoculation and 100 percent of the dogs developed lesions while Poste and King (90) reported that only 1 of 3 bitches inoculated intravaginally developed lesions and these lesions developed 7 days postinoculation.

Poste and King (90) did not report on any histopathological studies of these vaginal lesions so it is not possible to determine if the lesions they reported were lymphoid nodules rather than vesicles. It is highly probable that the lesions these authors have described are quite similar to the genital lesions experimentally produced in this study. However, it is interesting to note

that Poste (89) has reported that even though his isolate (CHV-BR) was serologically similar to CHV F-205 on the basis of neutralization and immunofluorescence tests there were marked differences between these two isolates by virtue of the CPE produced in DKC. Unfortunately it was not possible to obtain the CHV-BR strain so that pathogenicity comparisons could be made. In MDCK cells CHV-270 and CHV F-205 produce a similar CPE and an approximately equal number of intranuclear inclusions. It is therefore reasonable to assume that CHV-BR also differs *in vitro* with CHV-270 as well as differing *in vivo* as described previously. Poste (89) has noted the emergence of a non-syncytial CPE variant that produces CPE identical to that described for other CHV isolates.

It is interesting to note that all three of these isolates, CHV F-205, CHV-270, and CHV-BR have been isolated from different anatomical sites of dogs with different clinical conditions. Poste (89) has hypothesized that like *H. hominis* the site of isolation may affect the type of CPE produced.

Immunological and Epidemiological Aspects of CHV

All the dogs in this study inoculated with CHV-270 developed circulating neutralizing antibodies, which ranged in titer from 4 to 64, by day 28 postinoculation. The dogs in experiment 5, which were inoculated with CHV-33

by the intranasal and subcutaneous routes, developed similar antibody titers but the 4 dogs inoculated intravaginally failed to develop any neutralizing antibodies. These 4 dogs did however develop vaginal lesions and virus was reisolated for 6 days postinoculation from the vagina only, indicating that the infection only involved the genital tract. Upon challenge with CHV-270, neutralizing antibodies developed in the 4 dogs previously inoculated intravaginally with CHV-33, and again the virus was only isolated from the vagina. It would appear from these results that CHV-33 was less antigenic than CHV-270 at least in regard to stimulating humoral antibodies from a vaginal infection. After challenge with CHV-270 it became evident that CHV-33 had provided a degree of protection but this protection was incomplete. The protection could not be attributed entirely to circulating antibody because the 4 dogs inoculated intravaginally with CHV-33 did not develop circulating antibodies yet they were partially protected when challenged with CHV-270. It is possible that local secretory antibody (IgA) from the vaginal mucosa was stimulated by the CHV-33 infection thus providing some protection to subsequent CHV vaginal infections. Protection was based on the lack of spread of the virus in the dog and the lack of vaginal lesions. The challenge virus did not spread from the vagina in the 12 dogs pre-

viously inoculated with CHV-33 as compared to the control dogs from which the challenge virus was isolated from the conjunctiva, nasal cavity and the pharynx as well as the vagina. With respect to the development of vaginal lesions after challenge, only 4 of 12 dogs previously infected with CHV-33 developed lymphoid nodules characteristic of a CHV vaginal infection, whereas the control dogs in experiment 5 and all the dogs in experiment 4 developed vaginal lesions, including lymphoid nodules, when inoculated with CHV-270.

Low levels of circulating antibodies (4 to 16) in the dogs in experiment 7 did not seem to protect them from CHV respiratory infections. However, high-level antibodies protected against CHV respiratory disease as indicated by the fact that one dog with an antibody titer of 256 did not develop the respiratory symptoms which the other dogs developed when exposed to the lowered environmental temperature.

Having established that CHV-270 is a pathogen of the respiratory tract, the genital tract and occasionally the conjunctiva and that previous exposure to CHV can have an effect on subsequent infections, it is interesting to examine the whole CHV disease complex. The fact remains that the most important aspect of this virus in the dog population is in its ability to kill neonatal dogs.

CHV respiratory, genital and conjunctival infections are important primarily as a means of maintaining and transmitting the virus in nature. These three conditions caused by CHV could also be important to the veterinary diagnostician in the differential diagnosis of disease in individual dogs in spite of the fact that under normal environmental conditions the conjunctivitis, genital and respiratory tract diseases would be self-limiting. CHV infections may also cause stillbirths, abortions and infertility, but these are conditions in which CHV has been incriminated by only one group of investigators to date (90). If a CHV infection were to occur in a dog colony, breeding establishment or any premises where large numbers of dogs are bred and housed, the situation could potentially be very serious. For example a stud dog in a breeding colony with a CHV infection could infect several bitches resulting in the death of pups born subsequent to the infection. Poste and King (90) found that the CHV infections recurred so it is possible that with both viral latency and recurrence it would be impossible to rid a colony of CHV short of depopulating.

It would be possible to have a carrier bitch in a population that had normal litters of pups while acting as a source of infection for other dogs in the colony. Huxsoll and Hemelt (57) suggested that it was maternal anti-

bodies that protected these pups born to bitches that had previous litters die of a CHV infection. A situation could occur where the stress of whelping could cause a recurrence of a CHV infection in a carrier bitch but the litter would be protected by maternal antibodies. Thus the bitch would go unnoticed as the source of the infection.

Any indication that a colony of dogs has had contact with CHV, such as the presence of circulating neutralizing antibodies, would make one wary of the possibility of CHV infections in neonatal pups. However, the results of serological tests on day zero in experiment 7 indicate that all 8 dogs had either had CHV infection or possessed maternal antibodies passed from their dam. Although no determinations have been made of how long maternal antibodies to CHV are maintained, it seems unlikely that such titers would last 10 weeks. Regardless of whether the antibodies were stimulated in the pups or were maternal, CHV had to be present at some time in the commercial colony from which these dogs were obtained. The owner (53) of this colony reported that all dogs that died in the colony were routinely necropsied and no lesions typical of CHV infections in neonatal dogs had been noted. Furthermore, no increase in neonatal deaths had been reported. It was also reported by the owner that a recent check showed that 17 of 20 serum samples from producing bitches were positive

for CHV antibodies.

One can suggest many reasons why this colony has dogs with CHV circulating antibodies without neonatal deaths. For example these titers could be due to a closely-related virus that has yet to be discovered or possibly there are inapparent CHV infections in the colony that are caused by less pathogenic strains of this virus. Yet another possibility is that the environmental conditions in the colony, primarily the temperature, are such that infections in neonatal dogs do not progress to the point of a clinical disease.

One can easily ascertain that there are many unanswered questions about CHV especially with regard to latent and recurring infections, inapparent infections, and the role of circulating antibodies in a population of dogs. The recent findings by Poste and King (90) and the findings reported in this study add the role of CHV in genital infections and the resultant infertility and abortions to the list of areas that need to be more completely elucidated.

SUMMARY

This study was designed to determine the disease-producing potential of CHV-270 in non-neonatal dogs with particular emphasis on the respiratory and genital tracts. CHV respiratory infections were studied in dogs infected with CHV-270 and maintained at normal environmental temperatures as well as dogs infected and maintained at varied environmental temperatures. Experimentally-infected dogs were observed for clinical signs and sampled periodically to determine if CHV infection had become established and to what extent CHV was shed from the infected dogs. CHV antibody titers were monitored to determine the immunological response to the CHV infections.

Several interesting observations were made from these experiments. One of these observations was that with an added environmental stress, specifically maintenance of dogs at cyclic environmental temperatures between 0 C and 22 C, dogs experimentally infected with CHV-270 developed more severe signs than infected dogs maintained at a normal environmental temperature (22 C). Dogs maintained on the variable temperature regimen developed clinical signs of upper respiratory disease such as sneezing and severe rhinorrhea. Two such dogs died and upon postmortem examination a purulent rhinitis and conjunctivitis was present and there were foci of pulmonary hemorrhage.

Although B. bronchiseptica was isolated from the lungs of both of these dogs it was not considered a primary etiological agent. B. bronchiseptica was considered a secondary bacterial invader since subsequent experiments showed that dual infections with B. bronchiseptica and CHV-270 were no more severe than CHV-270 infection alone.

It was concluded that the lowering of the environmental temperature probably caused a lowering of the temperature of the respiratory tract epithelium to a temperature that was closer to the optimum for CHV replication, thus allowing the virus to replicate optimally and precipitate the clinical signs observed. The 2 dogs that died were in a group of 4 that, unlike other dogs infected with CHV and maintained at the variable environmental temperature, had lowered body temperatures. Again it was theorized that the lowered temperature allowed for greater CHV replication and thus the more severe clinical disease.

Another observation made from this study was the fact that CHV-270 caused a genital infection in both the male and female dog. The infection in the female was quite severe, however in dogs of both sexes the genital disease was self-limiting. Female dogs were primarily used for the study of the CHV genital infections because of the ease by which the lesions could be observed in comparison

to the difficulty of observing lesions in the male dog. The gross lesions consisted of several small glistening white raised areas in the genital mucosa with accompanying submucosal and petechial hemorrhages. Histopathological examination of the genital mucosa of both male and female dogs revealed that the raised areas were focal lymphoid nodules. These genital lesions could be consistently reproduced in female dogs not previously exposed to CHV.

Several dogs experienced a mild to severe conjunctivitis after intraocular inoculation of CHV-270 or after the spread of this virus to the eye. The mechanism of CHV spread within the dog is not completely understood. Viral spread from the respiratory tract to the genital tract, or vice versa, by licking is a probable mode of spread, but it is not the only mechanism of spread as was shown by the fact that dogs inoculated subcutaneously with CHV-270 shed virus from the oral and nasal cavities and the vagina.

The immunological response, as determined by the presence of circulating CHV-neutralizing antibodies, was consistently present after CHV-270 inoculation. CHV-33 stimulated an antibody response when inoculated subcutaneously and intranasally, but no circulating CHV antibodies were present after intravaginal inoculation. When these dogs were inoculated intranasally, intravaginally or subcutaneously with CHV-33 they were less susceptible to intravaginal challenge with CHV-270 than uninoculated dogs. However,

dogs that had moderate levels of CHV neutralizing antibodies (titers of 4 to 16) were as susceptible to CHV-270 inoculated intranasally as dogs with no antibody titers. One dog with a CHV antibody titer of 256 showed increased resistance to CHV-270 inoculated intranasally, however this dog did develop conjunctivitis and shed CHV. It appears that moderate levels of circulating CHV-neutralizing antibodies do not protect against CHV-270 respiratory infections but do partially protect against genital CHV infections.

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