# TRANSMISSION AND ATTEMPTED ISOLATION OF THE ETIOLOGIC AGENT ASSOCIATED WITH LYMPHOFOLLICULAR HYPERPLASIA OF THE CANINE SPECIES

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# TRANSMISSION AND ATTEMPTED ISOLATION OF THE ETIOLOGIC AGENT ASSOCIATED WITH LYMPHOFOLLICULAR HYPERPLASIA OF THE CANINE SPECIES

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## PREFACE

This study was concerned with the isolation and transmission of a viral agent associated with conjunctivitis, balanitis and vaginitis of the canine species.

The agent has been classified tentatively as a new member of the herpesvirus group.

Some investigators have attributed death in neonates as well as the above diseases to one and the same agent while others suggest the possibility of two different agents. The primary objective was to isolate the agent(s) in tissue culture and define its (their) role in diseases of the canine population.

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# CHAPTER I

## INTRODUCTION AND REVIEW OF LITERATURE

Viral agents have been associated with lymphofollicular hyperplasia in the canine species. The disease is characterized by granular conjunctivities, vaginitis and balanitis. In addition, neonatal death, stillbirth, abortion and infertility have also been attributed to infection by viral agents.

Recently agents associated with the above diseases have been shown to possess physical, chemical and biological properties consistent with the herpesvirus group. Neutralization studies using known herpesvirus antisera have ruled out identity with herpesvirus from other animal species; thus proving these agents to be new members of the group (1, 2). Certain of the isolates associated with neonatal death in the dog have been shown to be identical by plaque reduction tests (3). The serologic relationship of these viruses to those isolated from lymphofollicular hyperplasia has not been firmly established. In one study a similarity is reported (4), while in another study no relationship was shown (5).

The herpesvirus group is responsible for death and disease in several animal species. They have a broad vertebrate host range infecting man, domestic animals, and certain laboratory animals. Severity of diseases ranges from semi-latent infections to fatal ones. Diseases produced in fatally affected hosts consist of generalized

infections involving most organs and tissues including the nervous system (6, 7).

Generally, for each herpesvirus there exists a host(s) to which the virus is fatal, and a reservoir host(s) in which the virus produces only a sub-clinical or latent infection. For example, Herpesvirus suis is latent in adult swine and fatal in cattle; Herpesvirus hominis is latent in man and fatal in the owl monkey; and Herpesvirus simiae is latent in the rhesus monkey and fatal in man (8). The incidence of infection in the reservoir host is high, but overt disease is rarely seen. Lesions may develop and go unnoticed because they heal rapidly. Neutralizing antibodies are usually formed. The viruses are not usually eliminated from the reservoir host(s) and may be shed in secretions with only periodic recurrence of the lesions, usually as a result of various stimuli (8).

The experimental host range of the herpesvirus group is varied. The viruses of non-primates generally have a limited range and are also more species specific with regard to their natural host range, while those infecting primates have a broader experimental host range in that they may be propagated on a variety of tissue cell cultures and embryos of mice, chickens, rabbits, guinea pigs and hamsters. Moreover, these viruses affecting primates are not as limited with respect to the species of host affected in natural infections (8).

Classification of the group has evolved like other viruses from such characteristics as host range, cytopathology, and cytotrophism to more scientific physico-chemical properties. They are composed of double stranded DNA surrounded by a capsid which is icosahedral in shape and possessing 162 capsomeres. The complete virion, including the

envelope, measures 180-250 nm. They are sensitive to ether, heat, and acid. Studies of the antigenic relationship reveal the members of the group to be serologically distinct; however, some cross neutralization reactions have been demonstrated (9).

On the cellular level, Luria (10) reports that the overall response of infected cells can be either proliferative with apparent amitotic nuclear division and/or recruitment of nuclei into giant cells, or destructive. Polykarocytosis may be present in which cells coalesce, but individual nuclei appear to retain their identity. Transformation of host cells may also occur. The characteristic cytopathologic feature common to the group is the Cowdry type A intranuclear inclusion (8). This inclusion body is basophilic in its early stages but later becomes eosinophilic and separated from the nuclear membrane by a halo. These inclusions do not contain virus, they are merely empty shells which serve as evidence of viral multiplication. Herpesviruses are placed in two groups by some workers. Group A consists of those viruses which are readily released from cells in active forms, and Group B are those which are cell-associated with few, if any, free viruses in extracellular locations.

## Herpesviruses of Human-primates

Herpesviruses affecting humans are: H. hominis (herpes simplex), H. simiae (B virus), H. varicellae (varicella), H. zosteri (zoster) and H. suis (psuedorabies) (6, 7). The latter is reported to produce clinical manifestation of disease in man only rarely. H. hominis is the best known member of the group. A herpes-like virus with possible oncogenic propensity, was isolated from "Burkitt's lymphoma" associated

with Africans. The agent was assigned a passenger role since it persisted *in vitro* in the dividing cells for many weeks. The virus is believed to induce cell proliferation and hence to be of some significance in the etiology of the tumor (11, 12, 13). A viral agent, Herpesvirus type 2, has been recently associated with cervical carcinoma of the human female. The agent was shown to be venereally transmitted (14).

## Herpesviruses of the Equine Species

Among the herpesviruses isolated from horses, some are known to be pathogenic while others are apparently nonpathogenic. The three main groups are; equine herpesvirus I (EHVI), equine coital exanthema virus (ECE), and equine cytomegalovirus (ECM). The three types are widely distributed throughout the world, but only equine herpesvirus I has been well investigated. It is reported to have two serotypes with different geographical distribution. The usual clinical signs of equine herpesvirus I are respiratory disease and abortion in mares which may occur sporadically or in outbreaks (15). The virus that produces coital exanthema in the equine has been isolated from non-inoculated horse kidney cell cultures. In a study of venereal transmission, typical signs of coital exanthema were produced (16).

## Herpesviruses of the Bovine Species

A herpesvirus associated with rhinotracheitis, conjunctivitis, pustular vulvovaginitis, abortion, encephalitis and pustular balanoposthitis in cattle has been known since 1956. The virus was originally thought to be confined to the respiratory tract (17).

## Herpesviruses of the Avian Species

A herpesvirus causes infectious laryngotracheitis in chickens, a contagious disease characterized by inflammation of the larynx and trachea. A drop in egg production is seen in laying hens; and a weight loss in broilers. This disease was first discovered in the Rhode Island breed of chicken in 1925 (9). A herpesvirus was also found to be the causative agent of Marek's disease (neural lymphomatosis) in chickens. This agent has been placed in Group B due to its cell-bound nature (18). Herpesviruses have been isolated from normal turkeys. Parenteral inoculation of the agent into chickens results in protection against Marek's disease, hence has promise as a vaccine for chickens (19). Two strains of herpesvirus were isolated from racing pigeons suffering from a disease clinically similar to ornithosis. The isolates were found to be pathogenic for young pigeons, but not for chicks (20, 21).

## Herpesviruses of the Feline Species

In a survey of a cat population, for cytopathogenic agents of viral origin, Walton and Gillespie (22) isolated a herpesvirus from a litter of kittens suffering from an upper respiratory disease. The disease produced experimentally in kittens was extremely mild and an immunologic based protection against the disease was shown.

## Herpesviruses of Nonhuman Primates

Nonhuman primates are affected by seven known herpesviruses at this writing. The first isolation was by Sabin (7) in 1934 (B virus). This remained to be the only herpesvirus known to infect this group

until 1968. Since that time, due to the expanded use of these animals in biomedical research, the number has increased to seven.

## Herpesvirus of the Porcine Species

The porcine species serves as the natural host to a herpesvirus first reported in 1902 by Aujeskzy (9). The agent, first isolated from cattle, was found to produce a fatal infection in young pigs and a mild or latent infection in older ones. Several other animal species are infected by this agent, including man. The disease is commonly called Aujeskzy's disease or pseudorabies.

## Herpesviruses of the Canine Species

Herpesviruses affecting the canine species are the most recently described members of the herpesvirus group. One of the agents has been shown to pass the placental barrier, as has been shown with other animals, and remain latent in some fetuses while causing death in others. It is reported that some puppies acquiring the virus in this manner may carry it without ill effects if they survive the *in utero* infection (2).

In the fatal infections, there is generalized nephrosis of kidney tubules and massive interstitial hemorrhage of other organs, including lung, liver and brain. The histologic lesions seen in these fatal infections are reported to be disseminated necrosis with hemorrhage in the adjacent parenchyma. Severe necrosis of the renal cortex and the single intranuclear inclusion in areas of necrosis are said to differentiate it from canine distemper and infectious hepatitis (8, 23).

The first sign of the disease in experimentally infected puppies is a soft, odorless, yellowish-green stool which is seen in pups three to six days after intraperitoneal inoculation, and three to seven days after oral-nasal inoculation (24). In fatal infections, complete understanding of the disease in pups has been complicated by use of such terms as "fading puppy syndrome" and "cardiopulmonary syndrome." The two syndromes are reported to differ but may occur concurrently in the same animal with one condition predisposing to the other. The term "fading puppy syndrome" applies to puppies vigorous at birth but becoming progressively weaker and dying in three or four days. This term more accurately described the viral disease and is considered to be the one of choice. It is suggested, however, that such confusing terminology be avoided (25).

Motohashi and Tajima (26) reported that in 1959 they isolated a cytopathogenic agent by inoculation of canine kidney cell culture with long tissue from a three-year-old dog with severe respiratory disease. The long tissue was frozen (temperature not given) until 1966 at which time the agent was isolated and characterized as a herpesvirus. Pathogenicity studies were not done so it is not known whether the agent contributed to the above disorder. Isolation of a herpesvirus from a dog with clinical tracheo-bronchitis has been reported. The isolate caused respiratory disease when inoculated into older dogs (six months) and death when inoculated into four-day-old puppies. "Hemorrhagic disease" was said to be the cause of death (27).

Kakuk et al. (23) isolated a herpesvirus from a five-year-old dog with malignant lymphoma. The virus produced fatal septicemia when inoculated into neonates, but it has not as yet been shown to be

responsible for the malignant lymphoma.

Other investigators (28, 29) were unable to produce clinical disease in older dogs with agents isolated from natural cases of neonatal mortality. Severe disease in the young with mild or perhaps latent infection of the aged is a feature common to herpesviruses of certain other animals (7, 8, 9, 27).

A disease at present thought to be due to herpesvirus has been reported by Pickard (5). Follicles of lymphoid cells are present on the nictitating membrane, vagina and penis of both young adults and aged dogs, but primarily the young adult. The follicular lesions seen in the disease are said to regress and then recur later.

A recent report of a disease in older dogs believed due to herpesvirus was made by Poste and King (4) in 1971. The virus was isolated from vesicular lesions of the vestibule of the vagina in adult female dogs. The lesions were associated with histories of infertility, abortions, and stillbirths.

In regard to transmission, the most common mode for agents associated with neonatal death is believed to be during birth as puppies pass through the vagina of an infected bitch (25, 28). In utero infection, however, is known to occur (2). In older animals venereal transmission has been shown (4) and transmission from infected dogs to susceptible ones in close contact has been demonstrated (5, 23, 30).

Little information is available on the immunity developed by the dog to canine herpesvirus. It is reported to be poorly immunogenic (31). Antibody response in experimentally inoculated dogs is variable. Maternal antibody has been shown to protect puppies when the titers in the bitch were 1:4 or greater (3, 32). In two large kennels where

neonatal mortality was a problem, pooled serum, taken from several bitches that had lost litters, was inoculated intraperitoneally into pups one day of age. Losses were apparently halted. It was postulated that since pups are susceptible for only a short period, one inoculation should suffice (31).

The earliest report of a canine herpesvirus was published by Carmichael (24) in 1965. Subsequent to this report by Carmichael, viral agents shown by neutralization studies to be identical to Carmichael's original agent have been isolated from disease of fetuses and neonatal puppies (1, 2, 3, 23, 29, 32, 33). Cytopathogenic effect produced by the different isolates in canine kidney cell cultures was reported to be consistent. Moreover, all other tissue cells used namely, mouse, rat, horse, human, cow, pig, and chicken were resistant. Other characteristics of the virus were reportedly consistent with the herpesvirus group. Close structural relationships to herpes simplex virus were noted (33, 34, 35). Body temperature and its regulation is reported to be an important factor in the pathogenesis of the virus. Survival of the pups was shown to be prolonged when body temperature was elevated to 101° to 103°F. Conversely infection was enhanced by lowering the body temperature. It is suggested that high susceptibility of newborn animals may be due to both enhanced viral replication and impaired cellular or humoral response in the host (27, 31).

In one study, the isolate responsible for disease (respiratory) in older dogs was reported to cause death in neonates (27). In other studies, attempts to produce disease in neonates and older dogs with the same isolate were either not made or made without success (4, 5, 26, 28, 29). Two of these isolates (4, 5) were compared serologically with

Carmichael's agent. They were reported to be antigenically dissimilar.

The most recent report of a canine herpesvirus was made by Keller et al. (36). The agent was seen and identified as a herpesvirus by electron microscopy. Replication of the virus in tissue culture was achieved with difficulty.

Considerable work remains to be done with certain of these herpesvirus isolates from the canine species relative to antigenic identity and their role in the production of lymphofollicular lesions, infertility and abortion. Evidence presented to date fails to show conclusively that only one of the isolates is responsible for all of the previously described clinical manifestations in the dog. It has been well established that a herpesvirus can cause a fatal infection in the fetus and neonate, and that a herpesvirus has been unmistakably identified as the causative agent in the aforementioned disease of older dogs. However, investigations so far have shown that more than one strain of herpesvirus is responsible for disease in both young and old animals. As regards herpesvirus infections of certain other animals, including the human, it is well known that a given strain of virus may produce fatal infection of the young while producing only mild or latent infection of the aged (7, 8, 9, 27). Hence, it is not inconceivable that future investigations will reveal a similar pattern in the canine species.

## CHAPTER II

## MATERIALS AND METHODS

Minimum essential medium Eagle, with Hank's base without sodium bicarbonate, commercially prepared,[1] was used as tissue culture medium. The Eagle's medium was purchased in 100 ml. bottles of 10 X concentration. Fetal calf serum, also a BBL product, was used in 20 per cent concentrations for growth, and two per cent for maintenance. The culture medium was prepared by adding the contents of the 100 ml. 10x bottle to 877 ml. of sterile deionized water. To this solution, 4.7 ml. of 7.5 per cent sodium bicarbonate and 8 ml. of tricine buffer were added. Glutamine was added at the rate of one ml. (200 mM) dissolved in deionized water to 100 ml. of medium at time of use. Penicillin, streptomycin, and amphotericin B were added to the medium in concentrations of 100 u, 100 mcg. and 5 mcg. per ml. of medium respectively. The medium was dispensed in 100 ml. amounts and stored at $4^{\circ}C$ until used. One bottle (100 ml.) of each preparation was incubated at $37^{\circ}C$ and checked for bacterial growth. Fetal calf serum was usually added on the day of use. However, on several occasions serum was added at the time the medium was prepared. The medium described above was used initially with primary rabbit kidney cell cultures prepared

[1] BBL, Division of Bio Quest, Cockeysville, Maryland.

in this laboratory. When, after ten passages, no viral growth was evident, the medium was modified as follows: the calf serum concentration was reduced to 10 per cent, the penicillin and streptomycin concentrations were doubled, and use of the tricine buffer and the amphotericin B were discontinued.

Modified Earle's medium was also used for the primary rabbit cell cultures. The balanced salt solution (Appendix A) was prepared and stored at -25°C. The lactalbumin and sodium bicarbonate were both prepared and stored at room temperature. On day of use penicillin, streptomycin and nystatin were added to the medium in concentrations of 100 u, 100 mcg., and 100 mcg. per ml. of medium, respectively. Fetal calf serum was then added to the medium to give a final concentration of either 10 per cent for cell growth or two per cent for cell maintenance.

Saline A with trypsin and versene (Appendix B) was used as the digest solution in processing the lesions and rabbit kidney. The solution was prepared and stored at -25°C. On day of use it was thawed in 37°C water bath and added to 610 ml. of sterile dionized water. Temperature of the digest solution was maintained at 37°C during use. Penicillin, 1,000 u/ml. and streptomycin, 1,000 mcg./ml., were added to the digest solution used in processing the rabbit kidney cell.

## Cell Cultures - Established Line

HeLa cells were grown in screw capped tubes (16 x 125 mm). The tubes were sealed in the early part of this research with black rubber stoppers and in the latter part with screw caps. The cells were suspended in either Eagle's or Earle's media with 10 per cent fetal calf

serum, planted 2 ml./tube and incubated in a stationary position at 37°C. Spent medium was replaced with new growth medium every other day or as needed until confluency was reached.

Cell Cultures - Primary Line

Rabbit kidneys from 5-10 day old rabbits were removed and placed in a beaker containing digest solution which had been prewarmed to 37°C. The kidneys were cut in half longitudinally. The capsule and fat were removed and the cortex minced into 3-5 mm chunks. The minced tissue was then placed in a flask and washed by agitating in the digest solution and decanting until free of erythrocytes. After the final wash and decantation, 100 ml of digest solution was added back to the flask. The trypsinizing flask was then placed on a magnetic stirrer. The tissue suspension was allowed to digest at 37°C for 10-15 minutes and decanted. The supernate from this first digestion period was discarded. Digest solution (100 ml) was added back to the flask and 10-15 minute digestion periods and decantations were continued until disaggregation of the tissue was achieved. Decantation from each digestion period was filtered through a double layer of sterile gauze and collected in a cold flask. This flask was returned to the refrigerator after each decantation.

A pool of the supernates from the digestion periods was centrifuged at 900 rpm for 10 minutes at 4°C. The pellet was suspended in culture medium and washed a total of three times. The cells were centrifuged in the same manner after each wash. After the final wash, the cells were resuspended in 10 to 50 ml of medium and counted as follows: the suspension was diluted 1/10 with nigrosin (50 mg. nigrosin in

100 ml. Eagle's) in a leukocyte pipette (37). The preparation was allowed to incubate at room temperature for 10 minutes. With this stain, viable cells remain unstained while the dead cells become brown or black in color. A hemacytometer was then filled and the cells were counted. The total count and number of viable cells were calculated as follows: average number of cells/sq. mm x 10 (dilution) x 10 (depth of chamber) x 1,000 (conversion from $mm^3$ to ml.) = number of cells per ml. The per cent viable cells was calculated as follows:

$$\frac{\text{Total number of cells} - \text{dead cells}}{\text{Total number of cells}} \times 100 = \text{per cent viable cells}$$

An appropriate number of cells was removed from the original suspension by pipette and diluted with growth medium to achieve a concentration of $2.5 \times 10^5$ viable cells per ml. This suspension was then planted in tubes (16 x 125 mm) 2 ml./tube with tightly secured rubber stoppers. The tubes were incubated at 37°C in a stationary position. Spent medium was poured off and replaced with new after 24 hours and every other day thereafter until confluency was reached. Usually about one week was required for this. After removing a sufficient volume of the cell suspension for planting, the remaining suspension was diluted with Eagle's Medium containing 7.5 per cent dimethyl sulfoxide (stabilizer) to a concentration of $2 \times 10^6$ viable cells per ml. The suspension containing the stabilizer was distributed in screw capped tubes 2 ml./ tube. The caps were replaced tightly, and the contents forzen at -25°C overnight then stored at -70°C.

## Viability Studies - Rabbit Kidney Cells

Preliminary to isolation attempts, it was necessary to determine whether or not the tissue cells with stabilizer would remain viable throughout the investigative period when stored at -70°C. If these cells remained viable over sufficiently long periods of time, the number of 5-10 day old rabbits needed would be reduced considerably and the difficulty in obtaining rabbits of the desired age at the proper time would be avoided. More importantly, ready accessibility of cells from the same strain of rabbit throughout the investigation would be a most desirable feature. These cells were prepared as previously described in primary cell culture and stored, with stabilizer, at -70°C in screw capped tubes (16 x 125 mm). The tubes were removed from the freezer weekly, thawed rapidly in a 37°C water bath and counted as previously described. The per cent viable cells were calculated each week. The suspensions were then diluted to a concentration of $2.5 \times 10^5$ viable cells per ml. and planted. The monolayers were observed daily and the number of days required to reach confluency was noted. Commercially prepared[2] rabbit kidney cells and canine kidney cells were used in later isolation attempts. Rabbit kidney cells were shipped in basal medium Eagle's with five per cent fetal calf serum. Dog kidney cells were shipped in Hank's basal medium Eagle's with 10 per cent fetal calf serum.

Both rabbit kidney cells and dog kidney cell cultures were observed microscopically upon arrival and incubated in a stationary position at

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[2]Flow Laboratories, Rockville, Maryland.

37°C over-night. If monolayers were confluent, old medium was poured off and replaced with Eagle's medium plus two per cent fetal calf serum for maintenance. If further growth was indicated, Eagle's medium with 10 per cent serum was used until confluency was achieved.

## Donor Animals and Preparation of Tissues from These Animals

Donors consisted of 18 dogs of varying breeds and ages (Table I). These animals, with one exception, were brought into the Oklahoma State University Veterinary Clinic where lymphofollicular hyperplasia was noted on the nictitating membrane or the genitalium. Some of the animals had these lesions in both areas. The animals were anesthetized with Surital.[3]

Lesions from the nictitating membrane were surgically removed intact, except where only small scattered foci of proliferation were seen. In such cases the membranes were scraped to remove the superficial lesions and the debris removed by swab. In both methods, the membrane was freed of all visible lesions. Vaginal material was obtained by curetting. The affected area of the penis, just posterior to the bulb, was scraped with a curette and tissue fragments removed with a swab.

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[3]Sodium Thioamylal for injection N.F., Parke-Davis, Detroit, Michigan.

TABLE I

DONOR ANIMALS

| No. of Animal | Breed | Sex | Age | Source of Lesion | Date Received |
|---|---|---|---|---|---|
| 1 | Great Dane | M | 1 yr. | Eye[a] | 1/5/72 |
| 2 | Visla | F | 9 mos. | Eye | 2/4/72 |
| 3 | Collie | M | 7 mos. | Eye Scrapings | 2/26/72 |
| 4 | German Shepherd | F | 8 mos. | Eye | 2/27/72 |
| 5 | German Shepherd | M | 1 yr. | Eye Scrapings | 4/13/72 |
| 6 | German Shepherd | F | 6 mos. | Eye | 5/16/72 |
| 7 | German Shepherd | M | 8 mos. | Penis Scrapings | 7/3/72 |
| 8 | Same Animal as No.7 | M | 8 mos. | Eye | 7/10/72 |
| 9 | Blood Hound | M | 2 yrs. | Eye | 7/10/72 |
| 10 | Irish Setter | F | 6 mos. | Eye | 7/10/72 |
| 11 | Irish Setter | M | 6 yrs. | Eye | 7/12/72 |
| 12 | Irish Setter | F | 10 mos. | Eye | 7/12/72 |
| 13 | Blood Hound | F | 2 yrs. | Eye | 7/12/72 |
| 14 | German Shepherd | F | ? | Vaginal Scrapings | 8/29/72 |
| 15 | Mixed | | ? | Vaginal Scrapings | ? |
| 16 | German Shepherd | F | 6 mos. | Vaginal Scrapings | 9/21/72 |
| 17 | Mixed | F | Adult | Vaginal Scrapings | 11/5/72 |
| 18 | Afghan | F | 2 yrs. | Vaginal Scrapings | 11/16/72 |

Note: Specimens Nos. 7 through 13, and 15 through 18, were processed on date collected without freezing. Specimens No. 1 through 6 were frozen at -70°C on dates shown, thawed 6/2/72, trypsinized and inoculated into tissue culture. Specimen No. 14 was frozen on date shown, thawed 9/14/72.

[a]Lesions from the eye, unless otherwise specified, were removed intact from the nictitating membrane.

All specimens, whether intact lesions, curettings, or swabs were obtained by clinicians and placed in a screw capped tube containing 2 ml. of Eagle's culture medium. Tubes containing the medium were kept at 4°C. The refrigerated tubes were checked daily by the investigator for presence of specimens. If specimens were present they were either frozen at -70°C or trypsinized immediately and used as inoculum for tissue culture. Tubes containing swabs and scrappings were treated with penicillin 5,000 u/ml. and streptomycin 5,000 mcg/ml. for one hour at 37°C and inoculated on tissue culture. Intact lesions were minced into 3-5 mm chunks and trypsinized. Scrapings with 3-5 mm chunks of tissue were also trypsinized. The digest solution was allowed to remain in the trypsinizing flask for a one hour digestion period. The 10-15 minutes pour offs were not made in processing the lesions. The suspensions resulting from trypsinization were centrifuged at 3,000 rpm for 20 minutes at 4°C. The pellets were washed with culture medium and resuspended in 10-50 ml. of medium depending on size of pellet. Suspensions were again treated with 5,000 u of penicillin and 5,000 mcg of streptomycin as previously described and a portion (0.2 ml.) used to inoculate tissue cultures. The remaining portions were stored at -70°C in screw capped tubes for later use.

## Experimental Animals

Five puppies of mixed breeding were obtained from private homes at ages shown in Table II. Animals Nos. 3, 4, and 5 were litter mates. The bitch had been killed when they were four weeks of age. Each of the five animals was examined for the presence of lymphofollicular hyperplasia in the nictitating membrane and genitalium. The animals

without gross lesions were housed in isolated animal rooms where they remained throughout the experiment except for those occasions when they were brought into the examination room for inoculation and study.

TABLE II

EXPERIMENTAL ANIMALS

| No. of Animal | Breed | Sex | Age | Date Inoculated |
|---|---|---|---|---|
| 1 | Mixed | M | 5 mos. | 9/14/72 |
| 2 | Mixed | F | 2 mos. | 10/12/72 |
| 3 | Mixed | F | 10 mos. | 12/21/72 |
| 4 | Mixed | M | 10 mos. | 12/21/72 |
| 5 | Mixed | F | 10 mos. | 12/21/72 |

## Culture of Cells from Lesions

After the lesions were processed as previously described, certain of the cell suspensions (animals Nos. 8, 9, and 10) were counted, as described under primary cell culture, and planted ($2.5 \times 10^5$ cells per ml.) in tubes fitted with rubber stoppers. The monolayers were given the same treatment as the primary cell cultures until confluency was reached. Growth medium was then replaced with maintenance medium.

The monolayers were observed daily for cytopathogenic effect. After 10 days some of the monolayers were trypsinized off the glass and passaged. This procedure was repeated three times. The monolayers were observed for one week each time. Finally, the cells were trypsinized off the glass, counted and stored, with stabilizer at -70°C in a concentration of $2 \times 10^6$ cells per ml.

## Bacterial Cultures

Cultures for bacteria including mycoplasma were started on all specimens at time of planting in tubes. Thioglycollate broth and blood agar plates were used for the bacterial cultures. Hartley's PPLO broth overlay and Hartley's PPLO agar plates (Appendix C) were used for mycoplasma cultures.

Mycoplasma cultures were incubated at 37°C in 5 - 8 per cent $CO_2$ and held for one week before discarding as negative. Bacterial cultures were incubated at 37°C and discarded after one week. Cultures for bacteria were likewise done on certain of the inoculated and uninoculated monolayers which had undergone degenerative changes.

## Isolation Procedures

### Part I

Monolayers of each cell culture, HeLa and rabbit kidney cells, were inoculated in triplicate. The cells were grown in two sets each. Earle's medium was used with one set and Eagle's medium with the other. Specimens for this first isolation attempt, totalling 10 in number, had been collected from infected animals and stored frozen for as long as

one year in some instances. Some were trypsinized suspensions of tissue and cell culture from previous investigations (5), others were intact lesions from the eye and vaginal scrapings. The suspensions from previous investigations had been frozen in liquid nitrogen and stored at -70°C. Each monolayer was inoculated in triplicate with 0.2 ml. of the cell suspensions. The inoculum was allowed to run slowly over the monolayers after the spent medium had been poured off. The monolayers were then incubated for one hour at 37°C to allow for attachment of cells and adsorption of virus. At the end of this adsorption period the appropriate maintenance medium was added and the tubes returned to the 37°C incubator. Blind passages were made at 96 hour intervals by scraping the cells from the wall of the tubes with a rubber policeman. New monolayers were inoculated in triplicate with 0.2 ml. each of these cell suspensions. All specimens were carried through 15 passages each, then discontinued.

Part II

Rabbit kidney primary cells prepared in this laboratory were used in this second isolation attempt. Cells were prepared and stored as described in the viability studies. Cells were prepared in sufficient numbers to supply the needs of this investigative period. The cell suspensions were removed from the freezer at four day intervals to allow for scheduled 96 hour passages of the cultures. They were thawed rapidly in a 37°C water bath and diluted with culture medium to a concentration of $2.5 \times 10^5$ viable cells per ml.

Specimens Nos. 1 through 6 (Table I) were collected and frozen on dates indicated. They were all thawed, processed, and inoculated on rabbit kidney cell culture 6/6/72. Specimens Nos. 7 through 13 were processed and inoculated on rabbit kidney cell culture as received without freezing. Monolayers were inoculated in duplicate, and Earle's medium was not used in this isolation attempt. Cells were grown in Eagle's with 20 per cent fetal calf serum and maintained with two per cent fetal calf serum. Two-tenth ml. of trypsinized cell suspensions was used as before. Blind passages were made at 96 hour intervals by scraping inoculated monolayers from the tubes with a rubber policeman, and inoculating 0.2 ml. of these cell suspensions on new monolayers. This procedure was repeated 10 times for each specimen.

Part III

For this isolation attempt commercially prepared cultures of rabbit kidney cells and dog kidney cells were used. Specimens Nos. 1, 2, 3, 6, 8, 14 and 15 which had been stored at -70°C on dates indicated in Table I were thawed, processed as previously described and inoculated in duplicate on both rabbit and canine kidney cell cultures on 9/14/72. Specimens Nos. 16 through 18 were subsequently processed and inoculated on both rabbit and canine kidney cell cultures as they were received (Table I). All specimens were carried through 12 blind passages at 96 hour intervals except for Specimen No. 18. This specimen was carried through six passages only, then frozen at -70°C in 7.5 per cent dimethyl sulfoxide.

## Transmissibility Study I--

## Animal to Animal

Source of inoculum for this study was animal No. 14 (Table I). Vaginal scrapings had been obtained as previously described and frozen at -70°C in 7.5 per cent dimethyl sulfoxide for 16 days prior to this inoculation.

A five-month old male pup of mixed breeding designated as pup No. 1 (Table II) was anesthetized with Surital. The area of the penis just posterior to the bulb was scarified with sterile gauze until slight bleeding was noted. A sterile swab was then dipped into the tissue suspension which had been thawed rapidly in a 37°C water bath. The swab was then rubbed over the scarfied area of the penis. Posterior aspects of the nictitating membrane of both eyes were likewise scarified and inoculated. Rabbit kidney cell and dog kidney cell monolayers were also inoculated with 0.2 ml. of the suspension at this time. The eyes and genitalium were checked at weekly intervals for gross lesions. Twenty-eight days later, a four-week-old female of mixed breeding, pup No. 2 (Table II) was placed in the same cage with pup No. 1 to test for natural transmission. A period of two weeks was allowed for possible infection and disease to occur. Examinations were then made weekly for appearance of lesions. The pups were allowed to remain in the same cage throughout this study. The experiment was terminated 110 days after inoculation of pup No. 1.

## Isolation Attempts from Experimentally Infected Animals

Lesions from the nictitating membranes of both eyes of pup No. 1 and one eye of pup No. 2 were removed, processed as described previously in isolation procedures and inoculated on rabbit and canine kidney cell cultures. Specimens from pup No. 1 were carried through 10 passages. Specimen from pup No. 2 was carried through six passages and stored at -70°C for later use as inoculum in further transmission studies.

## Transmissibility Study II-- Tissue Culture to Animal

The specimens from dog No. 18 (Table I) and dog No. 2 (Table II), which had been carried through six passages in rabbit kidney cell culture and frozen at -70°C for two weeks were thawed in a 37°C water bath and used to attempt transmission of the agent from tissue culture. Three ten-weeks-old pups, one male and two females, of mixed breeding (Table II) were used for this study. The pups were anesthetized with Surital and the nictitating membranes of the right eyes were abraded with sterile swabs which had been dipped into the cell culture suspensions. The posterior portion of the bulb of the penis was inoculated in like manner. Swabs were inserted into the vaginal orifice of the females and rubbed repeatedly across the floor of the vagina to abrade the surface. Pup No. 4, a male, was inoculated with specimen from dog No. 18 (Table I); pup No. 3, a female, was inoculated with specimen from dog No. 2 (Table II). Pup No. 5, a female, was

inoculated with a non-inoculated cell culture suspension (all suspensions were rabbit kidney cells). The pups were kenneled in separate cages but in the same room. It should be mentioned that these pups, Nos. 3, 4, and 5, were housed for 72 hours prior to inoculation in the same room with the two previously infected pups, Nos. 1 and 2. They were then inoculated and placed in a separate room. The three pups were examined weekly until lesions appeared.

## Cytology

Cytology examination of cell suspensions from specimen of dog No. 18 (Table I), and dog No. 2 (Table II), was made at the time viral isolation attempt via rabbit kidney cell was started.

Smears prepared from cells in culture derived from lesions were stained with hematoxylin and eosin. The cells were scraped off the glass with a rubber policeman, centrifuged and the pellet smeared on a microscope slide and air dried. After fixing with Bouin's fixative for three minutes, the smears were washed and stained with hematoxylin for another three minutes. The smears were then washed and stained with eosin for five minutes, air dried and coverslipped using Permount[4] as a mounting medium. The smears were then examined microscopically for inclusion bodies.

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[4]Histological Mounting Medium, Fisher Scientific Company, Fair Lawn, New Jersey.

# CHAPTER III

## RESULTS AND DISCUSSION

### Viability Studies - Rabbit Kidney Cells

Weekly counts done on the frozen primary rabbit kidney cells showed no appreciable decline in the number of viable cells during the first three months of storage. Confluent monolayers of actively growing and replicating cells developed in about one week. This compared favorably with monolayers of cells derived from suspensions that had not been frozen at all. After three months of storage, the number of viable cells gradually declined in the next 30 days to 50 per cent of the count before freezing. During this time, monolayers required increasingly longer periods to develop confluency until finally two to three weeks were required. When the decline was first noted, dilutions were adjusted to yield the desired number of viable cells for planting. However, as dilutions were decreased, the dimethyl sulfoxide concentration reached toxic levels. Normally, dilutions of 1/10 were made of the thawed suspensions. This dilution resulted in desirable concentrations of cells for planting as well as safe levels of dimethyl sulfoxide. Perhaps the frozen and thawed aliquots of the cell suspensions could have been washed by centrifugation to reduce the dimethyl sulfoxide concentrations had it been necessary to propagate these cells after four months of storage. This study was prompted by the need for a convenient source of primary cell cultures. Information

is available in the literature on the viability of established line cells when frozen. Little information is given on the life span of primary cells under the conditions of this research (37).

## Culture of Cells from Lesions

Growth and development of these cells were comparable to that of the other primary cells used in this research except for the initial 24 to 72 hours after the first planting. During this period, the cells attached to the glass but appeared to remain static. Then they began to multiply, becoming confluent after about two weeks. On subsequent passages the cells proliferated at a much higher rate. Monolayers were maintained and observed for 10 days without evidence of cytopathogenic effect. Peeling from the glass was noted but was thought to be due to age of the monolayer. After the 10 day period, the cells were passaged for three times. These monolayers were maintained and observed for cytopathogenic effect for one week after each passage. When no cytopathogenic effect was noted, the cultures were discontinued.

Growing these cells in culture was considered worthwhile, in that if the virus was actually present in the lesions at the time, it was thought possible that viral replication would occur in these cell cultures. Based on the production of cytopathogenic effect, that did not occur. The investigator assumes that the virus was either not present in the lesions at the time or that it was present in the absence of cytopathogenic effect in culture. It is conceivable that one might demonstrate viral infection by interference technique in culture of these cells. Electron microscopy of the lesions as well as of the lesion-derived cells in culture might supply information as to status

of the infection in absence of cytopathogenic effect in HeLa cells, rabbit and dog kidney cell cultures.

## Bacterial Cultures

All cultures for bacteria were negative. These cultures were done for the purpose of testing the sterility of the cell suspensions used as inoculum for tissue culture, not for the purpose of isolating any bacterial agents that might have been present in the tissue.

## Isolation Procedures

### Part I

Results of this isolation attempt using HeLa cells grown in both Eagle's and Earle's media were negative based on production of gross and microscopic cytopathogenic effect. At no time during any of the passages was there any evidence of viral infection.

### Part II

No evidence of viral replication was apparent in these rabbit kidney cell cultures. The absence of cytopathogenic effect in the isolation attempts is not without precedent. Of the several investigators of canine herpesvirus, only one has reported viral replication in rabbit kidney cells (5). After failure in this second isolation attempt, the investigator considered the possibility of selecting for a resistant cell type in freezing and storing of the cells. It was for the purpose of ruling out this possibility that commercial cells were used in the third attempt. There was some hope that these non-frozen cells along with the alterations made in the culture medium

would duplicate the results of the above cited investigator.

Part III

Commercially prepared rabbit kidney cell and dog kidney cell cultures were employed in this final attempt to isolate the etiologic agent of lymphofollicular hyperplasia. The first passage of specimens Nos. 1 through 6 were mistakenly exposed to 42°C temperature overnight. These specimens were carried, nevertheless, through 10 passages. Subsequent specimens were also carried through 10 passages. Of these later tissue specimens, Nos. 7 through 13 and Nos. 15 through 18 were considered to be choice material in that they were cultured fresh, as collected, without freezing.

There is some question as to whether the virus, if present, could have survived the 42°C temperature, however, only six of the 21 specimens were involved in the mishap. It is unlikely that only these particular specimens possessed virus. As to the absence of cytopathogenic effect in canine kidney cell cultures, this is not without precedent either. While most investigators have propagated the virus in canine kidney cells, in the most recent study extreme difficulty in propagating the virus in these cells was reported (35). Electron microscopy demonstrated presence of the virus in the absence of cytopathogenic effect.

The possibility of losing the virus in storage should not be excluded. As mentioned previously some of the specimens were frozen for considerable lengths of time before culture was attempted. It is not known whether or not this agent survives such storage. It should be noted, however, that on two occasions disease was produced,

indicating an infectious agent, by specimens which had been frozen for two weeks in 7.5 per cent dimethyl sulfoxide (see Transmission Studies).

### Transmissibility Study I--
### Animal to Animal

Experimental pup No. 1 (Table II) was examined on day five post-inoculation. Some erythema was noted at the inoculation sites on the nictitating membranes of both eyelids. There was some question as to whether this should be attributed to infection or trauma. On day eight post-inoculation the erythema was increased and it was obvious at this time that the condition was due to something other than scarification. On day 12 post-inoculation lymphofollicular hyperplasia was present on both membranes.

Lesions were not seen on the penis of this pup until day 84 post-inoculation when small lymphofollicular lesions developed at the inoculated site. While these lesions were quite small and few in number, they were considered significant in that they had not been seen on previous examinations. Subsequent examinations at weekly intervals revealed progression of the lesions until on day 100 post-inoculation they were found to be comparable in size and appearance to those seen in natural infections.

Twenth-eight days after infecting the above pup (pup No. 1) the second pup was placed in the same cage. This pup (pup No. 2) was examined at that time and no lesions were seen on the nictitating membrane or in the vagine. Thirty-three days later, lesions were seen

on the posterior aspect of both nictitating membranes of this animal. The animal was checked weekly and the lesions were seen to grow progressively larger.

The vagina of this pup remained without lesions until 77 days after exposure. At this time lesions were apparent on the floor of the vagina. It is of interest to note that the genitalium of pup No. 1 developed lesions 84 days post-inoculation and the genitalium of pup No. 2 developed lesions 77 days after initial contact with pup No. 1. Transmission by contact was shown to result in lesions in the same areas as those of the inoculated pup and the dogs seen in the clinic.

Venereal transmission was not a factor in this animal to animal study as the pups were sexually immature at the time they were infected. The penis of the male pup was actually scarified and inoculated, hence it was not surprising to find lesions there. But lesions on the genitalium of the contact pup (pup No. 2) in the absence of coitus suggests that the virus produces a viremia and that it may have a predilection for genital tissues as well as conjunctiva. Results of these transmission studies were similar to those reported by Pickard (5).

## Isolation Attempts from Experimentally Infected Animals

Twelve days post-inoculation the lesion on the nictitating membrane of the left eye of pup No. 1 (Table II) was removed, processed as described in isolation procedures and inoculated on primary rabbit and canine kidney cell cultures. Three days later the lesion on the nictitating membrane of the right eye was in like manner removed and

inoculated on primary rabbit and canine kidney cell cultures. Both specimens were carried through 10 passages. No cytopathogenicity was evident in any of these cultures. One isolation attempt was made from the left nictitating membrane of pup No. 2 (Table II) 36 days after exposure. The lesion was removed and processed in the same manner as those from donor animals and inoculated on primary rabbit and canine kidney cell cultures. The cultures were carried through six passages without evidence of cytopathogenic effect. These monolayers in the sixth passage were scraped from the glass with a rubber policeman, pooled, and frozen at -70°C in 7.5 per cent dimethyl sulfoxide. This material was used later as inoculum for other experimental animals in Transmissibility Study II.

No attempts were made to isolate the virus from the genitalia of these puppies. Lesions seen in these experimentally infected animals were similar to those seen in the natural infections and those described by other investigators (4, 5).

Consideration should perhaps be given to the possibility of initiating viral replication in tissue culture by manipulating pH and temperature. Attention might be given to age of the lesion and timing of isolation attempts. Virus may be present only at certain stages during development of the lesion.

## Transmissibility Study II--

### Tissue Culture to Animal

Animals used in this study were Nos. 3, 4, and 5 (Table II). The first week after inoculation, all nictitating membranes, including the control, appeared inflamed. The inflammation was attributed at this time to trauma, particularly since the control animal (pup No. 5) was also affected. On subsequent examinations, tissue changes were minimal with the control animal showing as much change as the test animals over a period of four weeks.

The animals were then left for five weeks before they were examined again. On this examination, erythema was present at the inoculation site on all membranes, including the control animal. However, animal No. 3, which was inoculated nine weeks previously, had developed typical lymphofollicular, granular appearing lesions on the nictitating membrane of the left eye. It should be recalled that material used in this inoculation was obtained from experimental pup No. 2 (Table II) and carried through six passages in primary rabbit kidney cell cultures and frozen at -70°C for two weeks prior to the inoculation. Attention should be called to the fact that only the right eye of each animal had been inoculated. The left eyes were checked first at each examination but not otherwise disturbed. Tissue culture had been discontinued by this time so no attempts were made to culture this lesion. The genitalia of all animals had remained negative up to this time.

The next examination was made on day 90 post-inoculation. The experimental animals used in earlier trials developed lesions in the genitalia at about this time after infection so the examination was

scheduled to coincide with the time required for appearance of lesions in those animals. The nictitating membranes of the control animal (No. 5) and test animal (No. 4) appeared essentially the same as before with erythema persisting but no lymphofollicular hyperplasia was evident. The genitalia of these two pups were without lesions. The proliferative lesions seen on the left nictitating membrane of pup No. 3 on the previous examination were definitely considered to be lymphofollicular hyperplasia at this time. The lesions had progressed to a granular lymphofollicular mass measuring 1 to 1.5 centimeters across. A few minute lesions were seen on the floor of the vagina of this animal. It is not clear why the nictitating membrane of the left eye of pup No. 3 developed lesions. One might assume that a viral agent was transmitted in this case. A further assumption might be that a viremia ensued with localization of the agent in the membrane of the left eye and subsequently the genitalium, but why no proliferative lesions occurred in the right eye remains to be one of the mysteries of this investigation. These animals have all been retained for further study.

## Cytology

Hematoxylin-eosin stains of the cell cultures made from lesions were void of any change indicative of viral multiplication. The cell suspension of specimens from dog No. 18 (Table I) and dog No. 2 (Table II) were submitted to the Oklahoma State University Veterinary Pathology Department for cytological examination. The specimen from dog No. 18 was reported to be negative for changes suggesting presence of a virus. The specimen from dog No. 2 was reported to contain many

mature lymphocytes and large reticulum type cells. One cell, of germinal epithelial or reticulum type, was found to contain a red intranuclear inclusion (Figure 1). No chromatin was seen within the nucleus and the cytoplasm was reportedly sparse green and granular (Sano's method of Pollak's trichrome stain). The cell size was said to correspond with either a germinal epithelial type or a reticulum cell. Differentiation between these two was reportedly not possible due to loss of normal morphology. One cell, in this instance, which contained an intranuclear inclusion body offers some indication of the presence of a herpesvirus as was reportedly isolated from a similar proliferative lesion of the canine by Pickard (5). Since typical lesions of the disease were produced in a dog inoculated with this material (after six passages in rabbit kidney cell culture) an infectious agent is believed to have been present.

Result of this research, relative to cytopathogenic effect in rabbit and canine kidney cell cultures, are in conflict with those reported by Pickard (5) and Poste et al. (4). However, variability of cytopathogenic effect in tissue culture is not incompatible with a herpesvirus; therefore, these results may, in an indirect way, concur with the findings of these investigators.

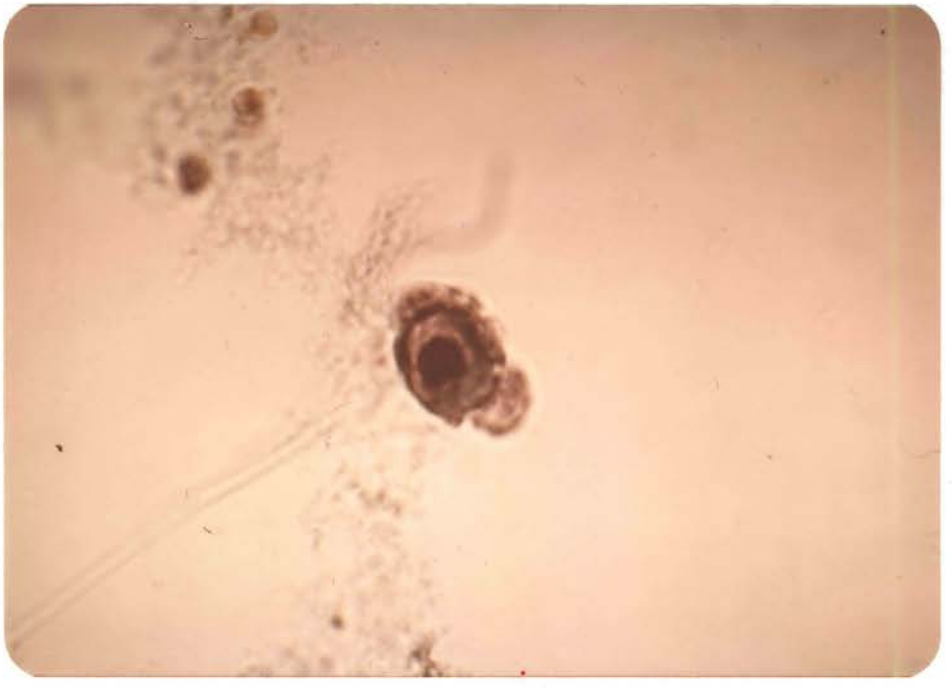

Figure 1. Cell Suspension of Specimen from Dog No. 2 (Table II)

# CHAPTER IV

## SUMMARY AND CONCLUSIONS

Attempts were made to isolate, in various cell cultures, a viral agent associated with lymphofollicular hyperplasia of the nictitating membrane and genitalium of the canine species. HeLa cells, rabbit kidney cells, and canine kidney cells were used in the isolation attempts. No cytopathogenicity was observed in any of the cultures inoculated with test material. Cell suspensions of certain of the lesions were grown in cell culture and observed for abnormalities but none was noted.

Characteristic lesions were reproduced in susceptible animals by inoculation and by contact with infected animals. The agent was found to be infectious for a dog after six passages in tissue culture (rabbit kidney cells) despite the absence of cytopathogenic effect.

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# APPENDIX A

## EARLE'S TISSUE CULTURE MEDIUM

## SCHEDULE FOR PREPARATION OF TISSUE CULTURE MEDIA

### Preparation of Nutrient Media

| Solutions | LE |
|---|---|
| 1. 10x Earle's salts | 100 ml |
| 2. 5% Lactalbumin | 100 ml |
| 3. 7.5% $NaHCO_3$ | 20 ml |
| Autoclaved distilled water | 780 ml |
| Total | 1 liter media |

Solution 1. Modified Earle's Balanced Salt Solution (10x)

| | g per liter |
|---|---|
| NaCl | 70.0 |
| KCl | 4.0 |
| $CaCl_2$ | 2.0 |
| $MgSO_4 7H_2O$ | 2.0 |
| Glucose | 10.0 |
| $NaH_2PO_4 \cdot H_2O$ | 1.4 |
| Phenol Red | 10 ml 0.2% stock solution (2cc of 1%) |

Distilled water to 100 ml

This solution is sterilized by filtration through 03 Selas or Seitz, dispensed in 100 ml volumes and stored at -18° F.

Solution 2. Lactalbumin enzymatic hydrolysate (L-H) 10x.

Fifty g are resuspended in 1 liter of distilled water, dissolved in steamer, filtered through coarse filter in a funnel and then filtered through a Seitz filter.

Solution 3. Sodium bicarbonate (7.5%) 50 x.

Seventy-five g are dissolved in 1 liter of distilled water, dispensed in 50 ml volumes and autoclaved at 15 pounds for 15 minutes. This solution can be stored in the refrigerator at room temperature in rubber stoppered containers.

# APPENDIX B

## SALINE A WITH TRYPSIN AND VERSENE

## Saline A

| | 1X | 10X |
|---|---|---|
| NaCl | 8.00 g | 30 g |
| KCl | 0.40 g | 4 g |
| Glucose | 1.00 g | 10 g |
| $NaHCO_3$ | 0.35 g | 3.5 g |
| (Double) distilled water | 1000 ml | 1000 ml |

Reference: Journal of Experimental Medicine 104(4): 615-628 (Oct. 1956).

This can be prepared in 10X concentration, dispensed in 100 ml amounts, autoclaved and frozen. Dilute with 900 ml of sterile distilled water before use. Add 250 units penicillin and 250 mcg streptomycin.

This is a calcium and magnesium free balanced salt solution used to wash cell sheet to remove media containing calcium and magnesium salts which interfere with the trypsin.

## Saline A with Versene* and Trypsin

1. Make up Saline A.
2. Prepare 1% trypsin solution.
3. Prepare 1% Versene solution.
4. Filter to sterilize--Seitz or Selas.

Add 50 ml of 1% trypsin solution to 1 liter of Saline A.
Add 20 ml of 1% Versene solution to above mixture.

Note: To prepare large amount of Saline A with Trypsin and Versene

Prepare: 1 liter of 10X Saline A
500 ml of trypsin
200 ml of Versene

This gives you a total of 1700 ml. Filter combined solutions through Seitz filter. Freeze in 170 ml amounts. To reconstitute, sterilize 830 ml of double distilled water in autoclave; cool. Then add bottle containing 170 ml of Saline A + T + V to make up to 1000 ml.

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*Versenes, Incorporated, Framingham, Massachusetts.

# APPENDIX C

## HARTLEY'S PPLO BROTH

## Hartley's Broth--PPLO Broth Media

| | | |
|---|---|---|
| Hank's "A" (10X) | 65. | ml |
| Distilled $H_2O$ | 585. | ml |
| Inactivated Swine Pathogen Free (SPF) serum ($56^{\circ}$ C for 30 minutes) | 333. | ml |
| Lactalbumin Hydrolysate (8.3 gm in 166 ml distilled $H_2O$ | 166. | ml |
| Yeast extract | 8.5 | ml |
| Phenol red (1.0% solution in distilled $H_2O$) | 1.6 | ml |
| $NaHCO_3$ (6.9% saturated solution) | 3.9 | ml |
| Hank's "B" (1000X) | .65 | ml |

(pH should be 7.2 - 7.4 check with pH meter)

Filter sterilize. Millipore filter or Selas filter.

Add:

| | | |
|---|---|---|
| Sterile Hartley's Broth (available commercially from COLAB Oxoid Product) | 500.00 | ml |

Can Add:

| | | |
|---|---|---|
| Penicillin--40,000 units per ml--at the rate of 0.5 ml/100 ml of above media | 8.3 | ml |
| Thallium acetate: 1:80 dilution at rate of 1.0 ml/100 ml of above media | 16.6 | ml |

# APPENDIX D

## HARTLEY'S PPLO AGAR

## Hartley's Meat Extract--PPLO Agar Base

| | | |
|---|---|---|
| Inactivated Swine Pathogen Free (SPF) serum ($56^{\circ}C$ for 30 minutes) | 333. | ml |
| 5% Lactalbumin hydrolysate (0.3 gm in 166 ml distilled $H_2O$) | 166 | ml |
| Yeast extract | 8.5 | ml |
| Phenol red (1.0% in distilled $H_2O$) | 1.6 | ml |
| 6.9% saturated solution of $NaHCO_3$ | 3.9 | ml |
| Hank's "B"(1000X) | 0.65 | ml |
| Hank's "A" 10X | 65.0 | ml |

pH should be 7.2 - 7.4 check on pH meter (add INHCl if needed). Then filter sterilize with Millipore filter 0.2 μ; Selas 0.3; or Seitz 0.1 μ

**Add:**

| | | |
|---|---|---|
| Sterile Hartley's Broth (available from COLAB, oxoid product) | 500 | ml |

**Use:**

195 ml of above agar base to Add to:
(Warm agar base to $50^{\circ}C$ before adding to agar)

| | | |
|---|---|---|
| Distilled $H_2O$ | 108.0 | ml |
| Oxoid Ion Agar, No. 2 (),75% agar) | 2.25 | gm |

Autoclave Agar 15 lbs/15 min
Cool to $50^{\circ}C$

300 ml agar pour approximately 45 small plates (5-1/2 cm diameter)

Do not autoclave Hank's Balanced Salt Solutions. Filter sterilize with Selas, Seitz, or Millipore.

VITA

James Andrew Jackson

Candidate for the Degree of

Master of Science

Thesis: TRANSMISSION AND ATTEMPTED ISOLATION OF THE ETIOLOGIC AGENT ASSOCIATED WITH LYMPHOFOLLICULAR HYPERPLASIA OF THE CANINE SPECIES

Major Field: Veterinary Parasitology

Biographical:

Personal Data: Born in Wynnewood, Oklahoma, May 30, 1924, the son of Mr. and Mrs. W. E. Jackson.

Education: General Education Development Test for High School completed in 1948. Diploma awarded by Lincoln High School, Wynnewood, Oklahoma in 1949; received the Bachelor of Arts degree from Southwestern College, Winfield, Kansas, with a major in pre-medical sciences, in August, 1951; Medical Technology Internship at Kansas City General Hospital, Kansas City, Missouri, September 1951-September, 1952; completed requirements for the Master of Science degree at Oklahoma State University in July, 1973.

Professional Experience: Employed as a Medical Technologist from 1952-1955, William Newton Memorial Hospital, Winfield, Kansas; St. Mary's Hospital, Winfield, Kansas, 1955-1960; Ponca City Hospital, Ponca City, Oklahoma, 1960-1968. Employed as Laboratory Supervisor in Veterinary Microbiology 1968 to present.

Professional Organizations: Registry of Medical Technology.