A STUDY OF *KLEBSIELLA PNEUMONIAE* INFECTION IN THE UTERUS OF THE MARE

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A STUDY OF KLEBSIELLA PNEUMONIAE INFECTION

IN THE UTERUS OF THE MARE

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PREFACE

This study was undertaken to lay the groundwork for study of the host-parasite relationship of the genital tract infection of the mare by K. pneumoniae. It is felt that a better method of isolation of the organism has been developed, and that a better understanding of the course of the disease and its etiologic agent has been attained.

I would like to thank my wife, Roberta Carrie, who while contributing financially to the welfare of the family, offering moral support, and superbly caring for our son, found the time to type the rough drafts and final copy of this thesis, and contributed unselfishly in the blood, sweat, and tears spent in preparing this manuscript.

My appreciation goes to the College of Veterinary Medicine for the financial support of the project and the use of the physical facilities of both the school and the Pawhuska Research Station. I would also like to thank the members of my graduate committee for their help in the project and their time to critique this manuscript.

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INTRODUCTION

Incrimination of Klebsiella pneumoniae as an equine genital infectious agent has already been documented by previous work. Most of those publications have dealt with infections occurring naturally. This thesis will report results obtained from artificially produced genital tract infections of mares with Klebsiella pneumoniae type 68. The strains of K. pneumoniae used in this study were isolated from stallions with clinically diagnosed genital tract infections.

The research objectives for the study were to:

Produce a uterine infection in mares using a strain of K. pneumoniae isolated from the genital tract of a stallion.

Monitor the infection by bacteriologic sampling of the uterus and determine the longevity of the infection and the effect of the estrous cycle on the infection.

Determine if the production of humoral antibody occurred in mares infected with K. pneumoniae.

CHAPTER II

LITERATURE REVIEW

Klebsiella pneumoniae infection of the equine reproductive tract was first described by Dimock and Edwards in 1926. The organism isolated from mares was classified as Encapsulatus genitalium and set apart from the Friedländer bacillus[13]. This organism was later found to be a Klebsiella sp. of capsule type 2 (Friedländer B)[15, 31]. The transfer mechanisms cited were coitus with an infected stallion and use of contaminated examination instruments and hands which were contaminated by infected mares being examined along with the non-infected mares.

A number of other papers have been published attesting to the fact that Klebsiella can produce an infection in the uterus of the equine, and it has now been generally accepted that Klebsiellosis in the equine is a venereal disease; however, the bacterium can be transferred by mechanical means[26, 12, 27, 28, 6, 1]. The main importance of genital tract infection with K. pneumoniae in the equine is the detrimental effect the infection has on the fertility of breeding stock[27, 17, 33]. The publication by Dimock & Edwards[13] contains clinical case reports of barren mares which were found to be infected with Encapsulatus genitalium (Klebsiella pneumoniae). This work also described two instances of apparent venereal transmission, where infected mares had coitus with stallions and those stallions transmitted the organism by coitus (service) to other mares which subsequently developed metritis or failed to conceive.

Merkt, et al, cited instances of mares which had aborted, and the uterus of the mares were found to be infected with Klebsiella sp. These infected mares were known to have had coitus with a stallion which was also found to be infected with K. pneumoniae[26]. Crouch, et al, reported the case of a chronically infected stallion which was believed to have become infected by servicing infected mares. Various treatments with antibiotics failed and the stallion was subsequently killed and necropsied for further study[12]. Chronically infected stallions may be one reservoir for the organism and an important factor in the epidemiology of these genital tract infections. Another source may be the environment. Data published by Merkt, et al, suggested that the environment in which the mares were serviced enhances the incidence of K. pneumoniae infection[26]. They reported a decrease in infections associated with the breeding act when the area of the service was changed from a sawdust composition to a gravel composition. Supportive evidence of infection with K. pneumoniae related to the environment was found in a paper linking an outbreak of mastitis in cows to bedding composed of sawdust[29].

There are no clinical signs which are pathognomonic for infection by K. pneumoniae. In mares with severe cases of metritis there has been reported a profuse, mucopurulent genital catarrh, which was red-brown to yellow-brown in color, and less severe cases may exhibit little or no exudate; whereas stallions were often asymptomatic[12, 13, 19, 26].

The organism can be isolated from samples taken by swab of the cervix and the uterus of the mare; or by urethral swab, in both the mare and the stallion, and by prepucial swab, and culture of semen in the stallion[13, 26, 12, 33, 6, 27, 28]. The report by Dimock, et al, shows

good evidence that with the genital tract infection by K. pneumoniae the cervix and uterus were the sites of infection[13]. The inflammatory changes thought to be due to the infection were confined to the cervix and uterus. It was thought that the infection was confined to the mucous membrane, since specimens taken of tissue below it did not contain this organism. Crouch, et al, were able to isolate Klebsiella by specimens taken at post-mortum examination from the urinary tract of a chronically infected stallion[12]. Specimens taken from the prepuce, urethra, prostate, bladder, and left renal pelvis were found to contain K. aerogenes (K. pneumoniae). None of those sites showed histological evidence of tissue invasion, and it was concluded that the infection was confined to the mucous membrane lining of the genito-urinary tract which parallels the conclusion of Dimock and Edwards.

The incidence of Klebsiella infection of the equine is low when compared to the incidence of infection with Streptococcus spp., group C (Lancefield). In a survey of brood mares in England 3,705 mares were sampled by uterine swab between December, 1963, and June, 1964, and 694 of these samples were found to contain organisms commonly associated with disease in the equine[33]. Klebsiella spp. comprised 8 percent and Streptococcus spp. 49 percent of the potential pathogens isolated in that study. Merkt, et al, reported that Klebsiella spp. comprised 10 percent of potential pathogenic organisms isolated from bacteriologic samples of the uterus in a study of reproductive problems in Thoroughbred mares in Germany from 1961-1973[26] Klebsiella sp. was isolated in 5 percent of barren mares in a report by Dimock and Edwards, while Streptococcus spp. were isolated from 26 percent of the mares sampled and reported by Dimock and Snyder; both of these reports were based on a seven-year

study of 1,600 barren mares in Lexington, Kentucky.[13] Even though Klebsiella infections are reportedly of low incidence, these infections are of major importance in that once a horse becomes infected, in most cases the infection is refractory to in vivo treatment with antibiotics to which the organism was found sensitive to by in vitro testing[12, 13, 26].

Data on the incidence of Klebsiella infection in horses in certain areas seems to point to an increase in that incidence over the past 10 years[26, 28]. This increase may reflect an actual increase in the rate of infection, but it is also likely that it represents an increased awareness and detection of Klebsiellosis. In any case, it is evident from the literature that in many European countries, veterinarians are becoming increasingly aware of K. pneumoniae as a cause of genital tract infections and its relationship to a decrease in fertility of equine breeding stock.

K. pneumoniae can be easily identified upon isolation. Bergeys' Manual of Determinitive Bacteriology for identification of microorganisms lists the necessary criteria for identification[7]. K. pneumoniae can be further classified as to capsule type using several serologic methods.

The capsule swelling test (Quellung, Neufeld reaction) is the simplest and quickest to perform and has been widely used[21, 16, 30, 4, 10]. The fluorescent antibody techniques[35, 36] and counterimmunoelectrophoresis are also used for identifying K. pneumoniae capsule types[22, 32].

There have been seventy-two distinct capsule types identified by using the capsule swelling test. These have been identified mainly by the work of Julianelle[20], Kauffmann[21], Edwards & Fite[16], Brooke[8], Edmunds[14], and Orskov[30]. The most frequently isolated capsule types reported to be associated with outbreaks of metritis in mares have been

2, 5, and 7[15, 31, 26, 12]. Stallions have been found to carry K. pneumoniae of types 2, 5, 7, 10, 30, and 68[15, 31, 26, 12, 37]. In stallions the question has been raised as to whether or not the capsule types other than 2 or 5 are pathogenic in the sense of causing infertility. Isolation of other capsule types from the prepuce of normal stallions without clinical signs of infection has not been uncommon[31, 26, 6].

CHAPTER III

MATERIALS AND METHODS

Experimental Animals

A Brief Statement on the History and Care of Mares in This Study

Pilot Study. Two mares of mixed breeding belonging to the Oklahoma State University (OSU) Veterinary School herd were used for the pilot experiment. Mares were on a ration of four pounds of an oat and corn mix[a] twice daily, and praire hay and water ad libitum. Both mares were between 10-15 years old and had previously produced live goals in the eight years they have been at OSU. Mare #2-S was in good health with no gross genital abnormalities. Mare #5-S was in good health but had a clinical history of genital tract infection with *E. coli* and *Streptococcus zooepidemicus*. In addition the mare had pneumovagina, probably caused by a damaged perineum which resulted from foaling, it was repaired one time, and with the next pariturition was reinjured and was not repaired. These mares were involved in the study which started January 30, 1976 and continued until May 10, 1976.

Principal Study. Eleven mares were used for this experiment. Their rations were the same as described for the mares used in the

[a]Ralston Purina, St. Louis, MO 83188.

pilot study. The mares were purchased from a sale barn in Paris, Arkansas, so their exact origin and previous background is not certain. Purchased and brought to the Veterinary School on April 20, 1976 were mares 1, 2, 7, 8, 9, 10, 437, and 559, while mares 3, 4, 5, and 6 were received May 14, 1976. These mares were housed together in an open pen, which had smaller holding pens for feeding and teasing. Their ages ranged from 3-10 years, and all but one, a shetland (#3), were of mixed breeding, with no mare having any gross genital abnormalities. All of the mares acquired in April developed clinical signs of strangles after arriving at the school. Only one, #559, had a severe case which required antibiotic treatment consisting of one 3 g dose of spectinomycin[b] given intravenously. Eight mares were transferred to the OSU Veterinary Research Station at Pawhuska, Oklahoma, on May 24, 1976. The other four mares (#7, 8, 9, 10) were transferred to a pasture eight miles west of the Veterinary School, and three of the four were later used as controls for the principal study as one mare died from exposure after becoming trapped in a mud hole. The eight mares were used in the principal study until August 30, 1976 at which time they were sold. The three control mares were used in the principal study until August 30, 1976 and were also sold.

Method of Tease

Pilot Study. The mares were teased daily during the course of the experiment. The tease was accomplished by leading the mares to the pen housing the stallion, and presenting the mares one at a time to the stallion. A fence separated the stallion from the mare. The behavior

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[b]Diamond Laboratories, Des Moines, Iowa 50304.

of the mare was observed for signs of an estrus period. Specifically, when the mare presented her posterior to the stallion, raised her tail, and assumed an inviting position with or without urination, and "winked"[c] to the stallion, she was noted as showing signs of an estrus period. Contact of the mare's genitalia by the stallion was kept to a minimum by keeping the mare just out of reach of the stallion. Nasal specimens were obtained from the stallion using dacron tipped swabs and cultured on May 12, and May 18, 1976.

Principal Study. While these mares were at the Veterinary School, the tease was performed using the same stallion as used in the pilot study. However, these mares were put in the holding pens which were in the main lot and the stallion was presented to each mare one at a time, and the mares were observed for signs of an estrus period as aforementioned. Upon transferring the mares to Pawhuska, a shetland pony stallion was then used for the tease. The mares were put into a lot as a group and the pony stallion was presented individually to each mare. The pony stallion was not tall enough to have contact with the genitalia of the mares. No nasal specimens were obtained from the pony stallion.

[c]"Winking" is a behavioral trait of mares in heat (estrus period), accomplished by muscular contractions of the clitoral area of the vulva which exposes the genitalia caudally.

Sampling Procedure

Tests Performed on Tampons to Determine Their Suitability for a Uterine Sampling Device

Three brands (T[d], M[e], P[f]) of regular size tampons without a deodorant were tested for their absorbancy of water. Three 250 ml glass beakers were used to hold 100 ml of distilled water for the test. Each tampon was immersed for 10 minutes in a separate beaker, lifted with forceps out of the water and held until it stopped dripping. The remaining water in the beaker was then measured in a 100 ml plastic graduated cylinder. This gave an indication of how much water the tampon would absorb and hold.

The tampons were checked for possible toxicity for the organism by making a 25 ml saline suspension of K. pneumoniae type 68 containing 1.00×10^3 org/ml and a 25 ml suspension of 1:100 dilution of that suspension containing 1.00×10^1 org/ml. The number of organisms in these suspensions was counted before and then after the tampon had been placed in the suspension for 10 minutes.

Method of Obtaining Uterine Specimens

Pilot Study. The mares were put into "H" frame stocks, and their tails were wrapped and tied forward. The vulva was thoroughly washed with full strength liquid soap[g] and rinsed with water until a wipe of

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[d] Tampax, Inc., Palmer, MA 01069.

[e] Cellulose Products Corp., Milltown, NJ 08850.

[f] Playtex Company, P. O. Box 1400, Dover, DL 19901.

[g] Phisohex®, Winthrop Laboratories, New York, NY 10016.

the vulva with a gauze pad showed that all debris and dried feces were removed.

On each sampling date the specimen from the uterus consisted of a method which utilized a tampon, and included a uterine biopsy obtained by a method utilized previously by Dr. R. M. Kenney, University of Pennsylvania, and has been outlined in a publication by Ricketts[34]. The biopsy forceps were disinfected at room temperature between samplings by scrubbing them in a 1:40 chlorhexidine[h] solution. A rectal exam glove was used on the scrub hand to protect the scrub solution from contamination from the hand, and after scrubbing the forceps were left in the solution for 10 minutes.

Two methods of tampon placement were used in this study:

Method A - After preparing the mare, an 18-inch, disposable cardboard tube speculum[i] was inserted into the vagina and the cervix was located using a flashlight as a light source. A tampon was fitted with two sterile strings, one taped to the applicator barrel, and one attached by a sheep bend knot to the tampon removal string. The tampon was held with the wrapping paper and was placed onto a long plastic rod so that the rod was against the backside of the tampon. The string on the tampon applicator barrel served to hold the tampon against the rod when tension was applied to the string. This apparatus was presented to the Os of the cervix via the speculum. With gentle manipulation, the applicator barrel was worked into the Os, and then by simultaneously pulling on the barrel

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[h]Nolvasan®, Ft. Dodge Laboratories, Ft. Dodge, IA.

[i]Alexander-Shaw Corp., Wellesley Hills, MA 02181.

string and pushing the rod, the tampon moved into the uterus. The rod and barrel were removed and the speculum, with the extended removal string handing out, was left in place. The tampon was left in place for 10 minutes and by realigning the speculum with the cervix, removal of the tampon was accomplished by pulling it back through the speculum, and after placing the tampon into a weighed 250 ml sterile plastic beaker[j] the excess string was cut off.

Method B - After preparing the mare, a plastic rectal exam glove was put on the hand of choice and a sterile latex surgery glove was carefully placed over that. The tampon was placed into the palm of the gloved hand and a sterile lubricant[k] was placed on the knuckles to lubricate the hand for insertion into the vagina. Care was taken to protect the end of the tampon in the palm of the hand during insertion to minimize contamination from the vaginal canal. Upon finding the cervix and then gently palpating it, the tampon could then be inserted through the OS and after expelling the tampon, the hand still holding the applicator barrel was withdrawn. After 10 minutes had elapsed the tampon was removed back through a disposable speculum by inserting the speculum and aligning the speculum with the cervix, then grasping the tampon string protruding from the cervix with the sterile biopsy forceps. The whole tampon was then placed into the sterile beaker.

Method A was used for about one-fourth the samples taken during the first part of the pilot study; thereafter, method B was used.

For the first seven samples a uterine specimen included the use of

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[j]Corning Glass, Corning, NY 14830.

[k]Johnson & Johnson, New Brunswick, NJ 08903.

a guarded culture swab[1] which was also used to obtain a specimen just prior to the placement of the tampon.

Principal Study. Mares were prepared for sampling as in the pilot study. One addition was that of using plastic liners in the wash buckets and changing the liners and water between samplings of each mare to reduce environmental contamination and to also reduce the possibility of spreading K. pneumoniae from mare to mare.

Specimens of the uterus were taken from the principal and control mares using Method B with the tampons[f] and included a uterine biopsy. The procedure for disinfecting the biopsy forceps after obtaining a specimen from a mare was modified as follows: (1) the forceps were first rinsed with water from a hose with a nozzle attached; (2) the scrub solution was changed to a 1:200 dilution of a quaterenary ammonium compound[m] (QAC); and (3) after a thorough scrub, the forceps were rinsed with sterile normal saline; (4) transferred to a one liter sterile glass cylinder filled with 70 percent (by volume) isopropyl alcohol; (5) the forceps were left in the alcohol for at least five minutes and when they were taken out of the alcohol for use on the next mare, they were (6) rinsed again with sterile normal saline.

A Test to Compare the Efficacy of Two Techniques for the Disinfection of Biopsy Forceps Contaminated with K. pneumoniae

A simple test was devised to determine if K. pneumoniae would

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[1]Haver-Lockhart Laboratories, Shawnee, KS 66201.

[m]Roccal-D®, Winthrop Laboratories, New York, NY 10016.

survive on the biopsy forceps after the forceps were scrubbed with and soaked for 10 minutes in the 1:40 chlorhexidine solution. The test was conducted at room temperature on May 21, 1976 while sampling the principal study mares #2 and #437, both of which had been inoculated 24 hr previously with K. pneumoniae type 68. After a uterine specimen was obtained from the mares, the forceps were used to inoculate 7 ml of eosin methylene blue broth, and then briefly rinsed with water, scrubbed, and left in the disinfectant for a period of ten minutes. After the time period had elapsed, the forceps were removed from the disinfectant and rinsed with sterile normal saline, and again used to inoculate 7 ml of eosin methylene blue broth. The tubes were incubated overnight at 37°C and the next day a sample from each tube was used to inoculate blood agar medium.

The same procedure was used to evaluate the efficacy of the disinfectant scrub coupled with a final soak of the biopsy forceps in 70 percent isopropyl alcohol. This test was performed on May 28, 1976 during the sampling of infected mares #2, #437 and non-infected mares #3, #4, #5, #6, and #559. All of these mares were used in the principal study.

Sampling Intervals for Taking the Uterine Specimen

Pilot Study. The mares were inoculated without respect to the estrous cycle since neither mare was out of the winter anestrus period at the time of initiation of this study. For the pilot study, the mares were sampled as follows:

a. A preinfection specimen from the uterus was obtained just prior to, and on the same day the mares were inoculated

intrauterinely with a K. pneumoniae suspension (January 30, 1976).

b. A 72-hr postinoculation (February 2, 1976) specimen from the uterus was obtained and thereafter specimens of the uterus were obtained weekly until May 10, 1976.

Principal Study. Except in the case of mares #6, #8, and #9, each mare was inoculated during an estrus period. Mare #6 was given a uterine douche of sterile normal saline prior to the week of June 26 to help stimulate a normal estrous cycle. The sampling intervals for obtaining the uterine specimens are given in Table I. The mares to be infected in this study were sampled as follows:

a. A preinfection specimen from the uterus was obtained by intrauterine placement of a tampon.

b. As each mare showed signs of an estrus period a specimen from the uterus was obtained and then the mare was inoculated intrauterinely with a K. pneumoniae suspension.

c. A 24-hr postinoculation sample was obtained, and thereafter weekly specimens from the uterus were obtained for one complete estrous cycle and then one more specimen was obtained the following week (Table I).

d. All the principal study mares excluding the controls were finally sampled on August 30, 1976 regardless of the date of inoculation.

The control mares in this study were sampled as follows:

a. A preinfection specimen from the uterus was obtained on August 16, 1976.

TABLE I

THE SAMPLING INTERVALS FOR MARES
IN THE PRINCIPAL STUDY

Mare	Week*[1, 2] 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	# Weeks post-inoc on Final Sample Date*[3]
1	June 18, 19	June 24	July 2	July 9	July 16			10
2	May 20, 21	May 27	June 4	June 11	June 18	June 24	July 2	14
3	June 3, 4	June 11	June 18	June 24	July 2	July 9	July 16	12
4	June 3, 4	June 11	June 18	June 24	July 2			10
5	June 3, 4	June 11	June 18	June 24	July 2			10
6	July 8, 9	July 16	July 23	July 30	Aug 5	Aug 16		7
437	May 20, 21	May 27	June 4	June 11	June 18	June 24	July 2	14
559	June 18, 19	June 24	July 2	July 9	July 16			10
7*[4]	Aug 16, 17	Aug 24	Aug 30					2
8*[4]	Aug 16, 17	Aug 24	Aug 30					2
9*[4]	Aug 16, 17	Aug 24	Aug 30					2

*[1] The number of weeks postinoculation.

*[2] The two dates under "Week 0" represent the inoculation and 24-hr postinoculation sample respectively.

*[3] August 30, 1976.

*[4] Control mares, all other mares were inoculated with K. pneumoniae.

b. On August 16, 1976, the uterus was infused with the sterile broth used in preparation of the inoculum.

c. A 24-hr postinoculation specimen from the uterus was obtained.

d. A 12-day postinoculation specimen from the uterus was obtained.

Inoculation of Mares

A specimen from the uterus was first obtained. A 60 cc sterile syringe, which had an 18 inch long sterile insemination catheter attached in place of the needle, was used to draw the inoculum from a bottle and transfer it into the uterus of a mare. The catheter was inserted into the uterus following the procedure for hand placement of a tampon into the uterus (Method B).

Pilot Study. The uterus of each mare in this study was infused with 25 ml of inoculum containing approximately 1×10^9 org/ml (an error in the serial dilution for the plate count prevented exact calculation).

Principal Study. The uterus of each mare to be infected for this study was infused with 26.7 ml of inoculum with concentration of *K. pneumoniae* as shown in Table II.

The uterus of the three control mares for this study was infused with 27 ml of sterile Worfel-Ferguson broth[n] on August 11, 1976.

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[n] Difco, Detroit, MI 48222.

TABLE II

DATE OF INOCULATION AND THE NUMBER OF K. PNEUMONIAE TYPE 68/ML OF INOCULUM PLACED INTO THE UTERUS OF EACH MARE IN THE PRINCIPAL STUDY

Mare	Date*[1]	Org/ml Suspension
1	6/18	3.90×10^9
2	5/20	2.20×10^9*[2]
3	6/03	3.15×10^9
4	6/03	2.25×10^9
5	6/03	2.80×10^9
6	7/08	5.80×10^9
437	5/20	3.50×10^9*[2]
559	6/18	2.50×10^9

*[1] 1976.

*[2] These mares received an inoculum that was kept at 2° - 4°C for 4 hr and the number shown is the plate count just prior to inoculation.

Bacteriology

Origin of the Organism Used in Preparation of the Inoculum

Pilot Study. The K. pneumoniae used in the inocula for the pilot study was isolated from the genital tract of stallions. The organisms were inoculated directly from the primary triple sugar iron (TSI) agar slant onto blood agar media (heart-infusion broth[n] with 2 percent agar[n]

and 5 percent bovine citrated blood). This blood agar medium was used throughout this study. The bacteria were then harvested after 24 hr incubation at 37°C, and lyophilized. Twenty-four hours before the mares were to be inoculated, the lyophilized bacteria were rehydrated and a loopful of the rehydrated culture was streaked onto blood plates for preparation of the inoculum.

The rehydrated culture was tested for purity by inoculating a medium of Worfel-Ferguson agar (broth with 2 percent agar) and checking the growth at 24 hr for mucoid, round, raised, entire colonies, of 3-5 mm in diameter. A capsule swelling test was performed on the bacteria to check for the homologous capsule type of K. pneumoniae.

Principal Study. The 72-hr postinoculation isolation of K. pneumoniae type 68 from mare #5-S which was passed once on blood agar, was used to inoculate the yolk sac of 7-day old chick embryos. The yolk was harvested in 24 hr (embryo death) using a sterile 3 ml syringe with an 18 gauge needle and then frozen in 1 ml aliquots and stored at -6°C. The day before each mare was to be inoculated, one of the 1.0 ml of frozen yolk aliquots was quickly thawed and a loopful streaked on enough blood agar media for preparation of the inoculum. The aliquots were tested for purity as in the pilot study procedure.

Preparation of the Inoculum

Two 18-hr blood agar cultures of K. pneumoniae each having 100 to 300 colonies were used for making 27 ml of inoculum in sterile Worfel-Ferguson broth. Sterile swabs were used to remove the colonies from the plates to a bottle containing the 27 ml of broth. The suspension was homogenized by repeatedly drawing the broth and organisms into and

ejecting them out of a 5 ml pipette. The suspension was either used immediately after taking out 0.3 ml for a plate count, or if the inoculation was delayed, the suspension was kept in a refrigerator and a sample for a plate count was removed just prior to inoculation of the mares.

Methods to Compute the Number of K. pneumoniae/ml of Uterine Fluid Collected From a Uterine Specimen

The amount of uterine secretion absorbed by the tampon was determined; the numbers of K. pneumoniae in the collected secretions was determined; and then the numbers of K. pneumoniae/ml of uterine secretion was calculated.

Determination of Amount of Secretion Absorbed by the Tampon. Each 250 ml beaker and tampon was weighed beforehand to an accuracy of 0.1 mg. After the tampon was recovered and put into the beaker, the beaker was reweighed. The weight of the wet tampon (Tw) was equal to the weight of the beaker and tampon (Bt) minus the beaker along (B). It was impractical to save the paper cover and the applicator for each tampon, therefore, a constant value of 2.76 g was used to account for these parts. Subtracting this corrected weight of the dry tampon (Td) from Tw equaled the weight of secretions that were absorbed. Assuming that 1 g = 1 ml, an approximate value for milliliters of secretion absorbed (Va) was determined.

Eq. 1. $Bt - B = Tw$

Eq. 2. $Tw - Td = Va$

In the pilot study the tampons were individually weighed only after week 6 postinoculation, when it was decided to determine an actual number

of K. pneumoniae of uterine secretion. Twenty tampons were weighed and a high and low range for the K. pneumoniae/ml of uterine fluid was determined for each sample by using the extremes of the tampon weight in the calculation.

Collection of the Uterine Secretion Sample from a Tampon. After the beaker containing the tampon used to obtain the uterine specimen was weighed, the tampon was removed from the beaker with forceps sterilized by passing it through a flame. A petri dish containing blood agar medium was inoculated with the tampon by smearing one-third of the surface of the medium with the tampon. The tampon was then put into a sterile 20 cc syringe which had a 16 gauge blunt needle attached. Five ml of sterile phosphate buffered saline^{o} was added to the syringe and this was allowed to absorb while the previously inoculated blood agar medium was streaked for bacterial isolation. The contents of the syringe was then ejected into a sterile tube marked "10^{o}" used in the ten-fold dilution series for the bacterial count. The tampon was then pulled from the syringe with sterilized forceps and placed back into the beaker. The contents of a screw cap tube containing 20 ml of enrichment broth was added to the beaker and this was incubated at 37^{o}C overnight.

Enumeration of K. pneumoniae in the Inocula and Secretions by a Plate Count Method. Blood agar was the medium used for the plate count method throughout this study. Ten-fold serial dilution of the sample was performed using sterile test tubes and pipettes, with sterile phosphate buffered saline as the diluent, in proportions of

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^{o}=0.85 percent NaCl dissolved in .01 M phosphate buffer at pH 7.4.

0.3 ml of sample in 2.7 ml of diluent. Sterile 1 ml disposable pipettes[j] were used for making the ten-fold dilutions, and reuseable 0.1 ml pipettes[j], graduated in .01 ml increments, were used to transfer six .01 ml aliquots of a dilution to a petri dish containing blood agar. The petri dishes were kept upright at room temperature for 30 minutes after inoculation, and then the plates were inverted and incubated at 37°C. Colony counts were performed at 12 hr for the inoculum and at 18 hr for the secretion.

Determination of K. pneumoniae/ml of Uterine Secretion. The six .01 ml aliquots were observed for growth after 18 hr incubation and the colonies counted in each aliquot. The dilution showing 10 to 100 colonies per .01 ml aliquot was used to determine an average number of colonies per .01 ml aliquot which was found by counting the six aliquots as a total and dividing that number by six. This number was multiplied by 100 and by the reciprocal of the dilution. The number found equaled the number of K. pneumoniae/ml of tampon wash (Kw).

Eq. 3. (avg # of K. pneumoniae)(100)(10^{x}) = Kw.

Since 5 ml of saline was used to wash the tampon, the total volume of the sample (Vt) was 5 plus Va (Eq. 2.). In order to find a value for the number of K. pneumoniae in the secretion, a dilution factor (D), to correct for the dilution of the secretion by washing the tampon, was found by dividing Vt by Va. The number of K. pneumoniae/ml of tampon wash multiplied by the dilution factor (D) gave the number of K. pneumoniae/ml of uterine secretion.

Eq. 4. (Kw)(D) = K. pneumoniae/ml secretion

Methods for Isolating K. pneumoniae From the Uterine Specimen

Standard microbiological testing for differentiation of bacteria cultured from the uterine specimens was employed. Six media were primarily used to differentiate K. pneumoniae from other gram negative, catalase producing, and non-cytochrome oxidase producing rod shaped bacteria isolated on the inoculated blood agar. These were: triple sugar iron agar (TSI)[n], glucose fermentation broth[n], citrate agar slant[n], urea agar slant[n], hydrogen sulfide-indole-motility agar (SIM)[n], and MacConkeys' agar[n] (Table III). Those organisms identified as K. pneumoniae were serologically identified as to capsule type by a capsule swelling method.

Eosin methylene blue broth was used as the sole enrichment and selective medium for the tampon used in collecting specimens in the first 10 sample dates (7 weeks) in the pilot study. On March 24, 1976 the methyl violet lactose broth[9] was also incorporated in the study as both a selective and enrichment medium for K. pneumoniae. The methyl violet lactose broth was used as the selective and enrichment medium for culture of the tampons used to obtain uterine specimens in the principal study. In both studies the enrichment media was added to the beaker containing a processed tampon as aforementioned. The following day a loopful of the medium was removed and used to inoculate blood agar media for isolation of organisms.

Capsule Swelling Technique

Worfel-Ferguson agar medium was prepared by adding 2 percent agar to Worfel-Ferguson broth. Organisms to be tested were streaked onto

TABLE III

REACTION K. PNEUMONIAE PRODUCES ON STANDARD MEDIA USED IN THIS STUDY

TSI	Glucose	Citrate*[1]	Urea*[2]	SIM	MacConkeys'
a/A	A	+	+	Non-motile	Mucoid,
No H_2S				No H_2S	Pink colony with dark pink center
				No indole*[3]	

+ - positive reaction.

a - acid.

A - acid and gas.

*[1] growth on slant with color change green to blue.

*[2] growth on slant with pink color change indicating hydrolysis of urea.

*[3] as detected using Kovac's test.

Worfel-Ferguson agar medium in petri plates and incubated 24 hr at 37°C. Enough organisms were taken from the Worfel-Ferguson agar and added to 3 ml of formolized (.5 percent) phosphate buffered saline to produce a wet mount containing 10-25 organisms per field at 970X.

One loopful of the suspension was mixed with one loopful of undiluted commercial capsular antisera[n] on a slide, and a cover slip was then put into place. At least five fields of the bacterial suspension were observed under 970X with subdued lighting for capsule swelling. A positive test was indicated by the formation of a line of demarcation around the capsule of the cell thus making it appear larger and giving

the organism the appearance of a donut. Brownian movement slowed down and the bacteria formed small aggregates. In a negative test, there may be some agglutination, but Brownian movement was not slowed. Cells retained a halo, but there was no line of demarcation around the capsule and no donut shapes.

Negative stains were made of organisms that did not react with any of the typing sera. The negative stain was accomplished by mixing a loopful of organism suspension with a loopful of nigrosin dye, and then a cover slip was drawn across the slide. This smear was allowed to air dry and then observed under 970X for encapsulated bacilli. Organisms not typeable by this researcher were sent to Ms. Mary Asbury, Center for Disease Control (CDC), Atlanta, Georgia for typing.

Method for Detection of K. pneumoniae in Biopsied Tissues Using a Fluorescent Antibody Technique

The conjugation of Klebsiella type 68 capsular antisera[n] with fluoroscein isothiocyanate[p] (FITC) was performed according to the method of Corstvet and Sadler[11]. The biopsied uterine tissue was frozen, sectioned into 6μ slices, allowed to dry at 37°C for 10 minutes on cover slips, and the sections fixed for 10 minutes in acetone, and then the sections were stained and observed according to the aforementioned methods.

Pilot Study. A combined total of 23 biopsy samples were collected from these mares, sectioned, and stained as aforementioned.

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[p]BBL Laboratories, Cockeysville, MD 21030.

Principal Study. Specimens obtained on the last sample date (8-30-76) were sectioned as aforementioned. Two sections of each specimen were stained with FITC conjugated antisera to K. pneumoniae type 68 and two sections of each specimen were stained with FITC conjugated antisera to Clostridium oediamatens[q].

Lyophilization of Bacteria

Klebsiella spp. as well as any other bacteria that were to be kept for future use were lyophilized as follows:

1. The 100 to 300 colonies of an 18 hr culture were transferred from the surface of blood agar medium in petri dishes with sterile swabs to a 16 x 125 mm test tube containing 3-5 ml of sterile, reconstituted, low fat dry milk and the suspension was mixed thoroughly;
2. 0.5 ml aliquots were then transferred from the milk tubes using a 1 cc syringe with a 25 gauge needle into sterile 5 ml lyophilization vials and shell frozen in 95 percent ethanol and dry ice; and
3. the bacteria were lyophilized, given an accession number, and stored at 4^{o}C.

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[q]Wellcome Laboratories, England.

Serologic Techniques

Testing of Mares Sera for Presence of Humoral Antibody to K. pneumoniae by the Capsule Swelling Technique

On each sample day, 8-10 ml of blood was collected from the neck vein using a vacutainer tube with an 18 gauge needle. Merthiolate (1:5000) was added as a preservative to 0.5 ml aliquots of the collected sera, and the aliquots were stored at $4^{o}C$. Starting with 0.1 ml of serum and using .1 ml of phosphate buffered saline as the diluent, a two-fold serial dilution series was made to a final dilution of 1:16. Each dilution of the serum was tested for ability to cause capsule swelling of the K. pneumoniae type 68.

Production of Immune Sera to Capsule Antigens of K. pneumoniae

Three white New Zealand rabbits, 2-3 months old, were used to produce antisera to K. pneumoniae types 2, 5, and 7.

A preinoculation serum sample was obtained from the rabbits and tested for its ability to cause capsule swelling of K. pneumoniae capsule types 2, 5, and 7. The rabbits were inoculated with 1 ml intravenously and 2 ml subcutaneously with a dense suspension (#3 McFarland) of formalin killed (.5 percent formalin for 48 hr) K. pneumoniae in phosphate buffered saline. Subsequent injections were made on day three and day six post initial inoculation.

Fourteen days after the first injection, 3 ml of blood was taken from an ear vein of each rabbit and the sera tested for capsule swelling

of homologous organism. Rabbits whose sera gave a positive test were exsanguinated by cardiac puncture. Serum was collected, aliquoted, and frozen at $-6^{o}C$.

CHAPTER IV

RESULTS

Sampling Procedure

Use of Tampon as a Uterine Sampling Device

Method A of tampon placement was used in the pilot study until March 4, 1976. This method was found to be unsatisfactory whenever the cervix was tight or lying on the floor of the vaginal vault. With these conditions, it was always necessary to hand place the tampon so that the cervix could be palpated with the fingers to prepare it for insertion of the tampon. Method B proved to be more satisfactory in that the assembly of an apparatus was not required as was with Method A. Method B was therefore less time consuming and did not require that the vaginal vault be exposed to the air for ten minutes as with Method A, since a speculum was used only for the retrieval of the tampon. When using Method A, it was found that if the mare urinated during the ten minutes in which a speculum was in place, the speculum itself provided a channel by which urine could contaminate the anterior vaginal vault.

Tampon T was used in the study until April 1, 1976 when at that time the tampon P was introduced. The tampon T applicator barrel would fall apart when it became saturated with mucus during insertion, therefore the cardboard applicator was found to not be waterproof. Two other brands of tampons with plastic applicators were then considered.

Tampon P was the most absorbant of the three tampon brands tested. They were found to be the most satisfactory for the hand placement method because the applicator barrel was plastic and its end was enclosed, therefore protecting the tampon. The plastic applicator barrels held together for the manipulation involved for insertion of the tampon through the cervix whereas the cardboard type became wet and subsequently lost their rigidity. The non-deodorant type of tampon was chosen to avoid the possibility of any bactericidal action of a deodorant. No significant changes in the number of K. pneumoniae/ml were noted when tampon P was placed into the saline suspensions of the organism.

Pilot Study. The results of the isolation of K. pneumoniae from samples obtained with a guarded culture swab were compared to the results of isolation of K. pneumoniae from samples obtained with a tampon in Table IV. A total of thirteen uterine specimens were compared and of these, nine K. pneumoniae isolations were from blood agar media inoculated directly from the tampon used to obtain a uterine specimen. There was no K. pneumoniae isolated from the blood agar media inoculated with the guarded culture instrument used to obtain the uterine specimen. There were eleven K. pneumoniae isolations made from the enrichment media inoculated with the tampon, whereas only three K. pneumoniae isolations were made from enrichment media inoculated with the swab from the guarded instrument.

Principal Study. In only one instance with mare #3 (4 week post-inoculation), was the tampon method of obtaining uterine specimen unsuccessful.

TABLE IV

ISOLATIONS OF K. PNEUMONIAE FROM UTERINE SPECIMENS OF MARES IN THE PILOT STUDY TAKEN WITH BOTH THE TAMPON AND THE GUARDED SWAB

Mare	Week	Date	Tampon		Swab	
			Direct*[1]	Enrich*[2]	Direct	Enrich
2-S	1	2-06	no	no	no	no
	2	2-11	yes	yes	no	no
	2	2-13	yes	yes	no	no
	3	2-18	no	yes	no	no
	4	2-25	no	no	no sample	
	5	3-04	yes	yes	no	no
	6	3-11	yes	yes	no	yes
5-S	1	2-06	yes	yes	no	no
	2	2-11	no sample		no sample	
	2	2-13	yes	yes	no	yes
	3	2-18	yes	yes	no	no
	4	2-25	yes	yes	no	yes
	5	3-04	no	yes	no	no
	6	3-11	yes	yes	no	yes

*[1] blood agar medium inoculated by direct placement of the instrument onto the surface of the medium and then the inoculated area was streaked for isolation of colonies.

*[2] enrichment media inoculated and incubated 24 hr at 37°C and a sample placed onto blood agar media and streaked for isolation of colonies.

Yes - K. pneumoniae isolated.

No - No K. pneumoniae isolated.

No sample - a sample by this method was not obtained on this sample date.

Efficacy of the Two Techniques Used for the Disinfection of Contaminated Biopsy Forceps

Each tube of eosin methylene blue medium inoculated on May 21, 1976 with the biopsy forceps washed with a 1:40 chlorhexidine solution after each biopsy of the infected mares #437 and #2 (from the principal study) showed signs of growth (turbidity) in 24 hr and were found to contain pure cultures of K. pneumoniae type 68. The uterus of both mares was inoculated 24 hr prior to the test with K. pneumoniae type 68. The eosin methylene blue media inoculated on May 28, 1976 with the biopsy forceps disinfected by the 70 percent alcohol technique were observed for growth at 24 and 48 hr. Only one of these tubes showed evidence of growth (turbidity) and was found to contain a large gram positive spreading bacillus. There were no K. pneumoniae type 68 isolated from these media even though mares #437 and #2 were still infected with the organism.

Inoculation of Mares

Pilot Study. Both of the mares in the pilot study were inoculated intrauterinely with K. pneumoniae and shown to be infected by subsequent reisolation of the organism from the uterine specimens taken postinoculation (Figure 1). Neither mare had a vaginal discharge in 24 hr and both remained asymptomatic throughout the remainder of the experiment.

Principal Study. The eight mares in this study inoculated intrauterinely with K. pneumoniae type 68 were shown to be infected by subsequent reisolation of the organism from the uterine specimens taken postinoculation (Figure 2). Three inoculated mares #437, #1, and #6

developed a yellow-brown mucopurulent vaginal discharge of small volume 24 hr postinoculation which was not evident 48 hr postinoculation. The mares remained asymptomatic throughout the rest of the experiment. The control mares remained asymptomatic even after inoculation with the sterile broth.

Bacteriology

Determination of the Amount of Secretion Absorbed by the Tampon Used in Obtaining a Uterine Specimen

Pilot Study. Quantitation of the amount of uterine fluid collected by the tampon was begun seven weeks postinoculation (March 19, 1976) and was continued until week 14 postinoculation (May 10, 1976). These data are given in Table V. The amount of uterine fluid measured in each mare ranged from 0.49 g to 1.71 g for #2-S and 1.08 g to 4.92 g for #5-S.

Principal Study. The amount of uterine fluid collected by the tampon was calculated for each postinoculation sample date of all the mares used in this experiment. These data are given in Table VI. The amounts of fluid collected from the 24 hr postinoculation specimens of the mares inoculated with *K. pneumoniae* varied from 2.40 g to 7.90 g. The amounts of fluid for the remaining number of uterine specimens collected from the inoculated mares ranged from 0.40 g to 5.20 g. The amounts of uterine fluid collected from the uterine specimen of the control mares did not vary significantly between the preinoculation, the 24 hr postinoculation, and thereafter; only one sample from one mare, #9, was greater than 1 g. The 24 hr postinoculation sample from mare #9 was heavily contaminated with lubricant and therefore the true value for that particular sample was impossible to accurately determine.

TABLE V

THE AMOUNT OF UTERINE FLUID COLLECTED AND DETERMINATION OF THE NUMBERS OF K. PNEUMONIAE/ML OF UTERINE SECRETION FROM MARES IN THE PILOT STUDY

Mare	Week	Date	Wt of Uterine Fluid Absorbed	K*[1] /ml of Wash*[2]	K/ml of Uterine Fluid
2-S	7	3-19	1.5524	1×10^2	4.2×10^2
	8	3-24	*[3]	0	ND*[4]
	9	4-01	1.7067	0	ND
	10-11	4-13	0.8745	0	ND
	12	4-22	0.9286	0	ND
	13-14	5-10	0.4086	0	ND
5-S	7	3-19	2.6815	1×10^2	4.2×10^2
	8	3-24	*[3]	1×10^2	4.2×10^2
	9	4-01	4.9181	0	ND
	10-11	4-13	1.0820	1×10^2	4.2×10^2
	12	4-22	3.9691	1×10^2	4.2×10^2
	13-14	5-10	2.04	1×10^2	4.2×10^2

*[1] K. pneumoniae.

*[2] Fluid flushed from the tampon using 5 ml of sterile saline.

*[3] Technical error; no measurement.

*[4] Not determined since no K. pneumoniae were enumerable by the plate count method.

TABLE VI

THE AMOUNT OF UTERINE FLUID COLLECTED FROM THE UTERINE SPECIMEN OBTAINED FROM MARES IN THE PRINCIPAL STUDY

Mare	Days Postinoculation								
	0	1	7	14	21	28	35	42	FS*[1]
1	0.98*[2]	3.92	1.12	1.61	0.72	1.27			1.07
2		3.09	2.05	*[3]	0.82	2.41	1.24	2.27	1.60
3		2.39	2.83	0.61	2.02	*[3]	2.19	0.77	1.23
4		7.89	1.44	2.78	0.64	1.03			1.57
5		2.69	0.71	0.55	0.60	0.40			1.20
6	0.82	3.35	1.08	0.77	0.37	0.74	0.74		0.65
437		3.52	3.26	1.15	1.09	1.42	5.15	1.23	0.56
559	1.52	4.30	0.62	0.44	0.89	0.55			0.58
7	0.92	1.08	6.27						
8	0.89	0.45	0.34						
9	0.81	9.58	0.12						

*[1] Final sample date, 8-30-76.

*[2] Measurements are in grams.

*[3] Technical error; no measurement.

K. pneumoniae/ml of Uterine Secretion as Determined by the Plate Count Method

Pilot Study. The numbers of K. pneumoniae/ml of uterine secretion for weeks postinoculation 7 through 14 were calculated and are presented

in Table V. Neither mare was found to have greater than 4.2×10^2 K. pneumoniae/ml of uterine secretion during or after week 7 postinoculation. The estimations of K. pneumoniae/ml of uterine secretion for the weeks 0 to 6 were calculated using two dilution factors which were determined using both the greatest and the least amounts of fluid absorbed by the tampons used in obtaining the uterine specimens from week 7 until week 14. The numbers of K. pneumoniae/ml of uterine secretion for the weeks 0 through 6 varied from 5.9×10^2 to 5.52×10^5 when the smaller dilution factor was used, and 1.2×10^3 to 1.5×10^6 when the larger dilution factor was used. These data are given in Table VII.

Principal Study. The results of the determination of the concentration of K. pneumoniae/ml of uterine fluid of each mare used in the principal study are given in Table VIII. The 24 hr postinoculation uterine specimens yielded 5.41×10^2 to 3.42×10^5 K. pneumoniae/ml of uterine secretion. Counts of K. pneumoniae from subsequent uterine specimens yielded 1.28×10^2 to 7.1×10^4 K. pneumoniae/ml of uterine secretion. No K. pneumoniae were enumerable by the plate count method from the uterine specimens taken on the final sample date (August 30, 1976), however, K. pneumoniae was isolated from the uterine specimens of mares #2, #4, and #6.

Isolation of K. pneumoniae From the Uterine Specimens

Pilot Study. The data for postinoculation isolations of K. pneumoniae from the uterine specimens of the mares in this experiment are given in Figure 1. K. pneumoniae was not isolated from either mare on the day of inoculation of the uterus. K. pneumoniae was isolated

TABLE VII

ESTIMATION OF K. PNEUMONIAE/ML OF UTERINE FLUID FROM MARES IN THE PILOT STUDY FOR SAMPLES TAKEN BEFORE WEEK 7 POSTINOCULATION

Mare	Week	Date	#K*[1] / ml Wash*[2]	Est. K/ml DF 1:3.94*[3]	Uterine Fluid DF 1:7.8*[4]
2-S	0	1-30	0	---	---
	0	2-02	0	---	---
	1	2-06	0	---	---
	2	2-11	0	---	---
	2	2-13	8.66×10^2	3.41×10^3	6.7×10^3
	3	2-18	0	---	---
	4	2-25	0	---	---
	5	3-04	9.2×10^2	3.62×10^3	7.1×10^3
	6	3-11	1.5×10^2	5.9×10^2	1.2×10^3
5-S	0	1-30	0	---	---
	0	2-02	*[5]	---	---
	1	2-06	2.76×10^5	5.52×10^5	1.5×10^6
	2	2-11	Not sampled	---	---
	2	2-13	1.7×10^4	3.40×10^4	9.5×10^4
	3	2-18	9.7×10^3	1.94×10^4	5.43×10^3
	4	2-25	1.38×10^3	2.76×10^3	7.73×10^3
	5	3-04	0	---	---
	6	3-11	1.68×10^4	3.36×10^4	9.31×10^4

*[1] K. pneumoniae.

*[2] Fluid flushed from the tampon using 5 ml of sterile saline.

*[3] Dilution factor determined from the greatest amount of uterine fluid absorbed by a tampon in this study (Table V).

*[4] Dilution factor determined from the least amount of uterine fluid absorbed by a tampon in this study (Table V).

*[5] Technical error; no measurement.

TABLE VIII

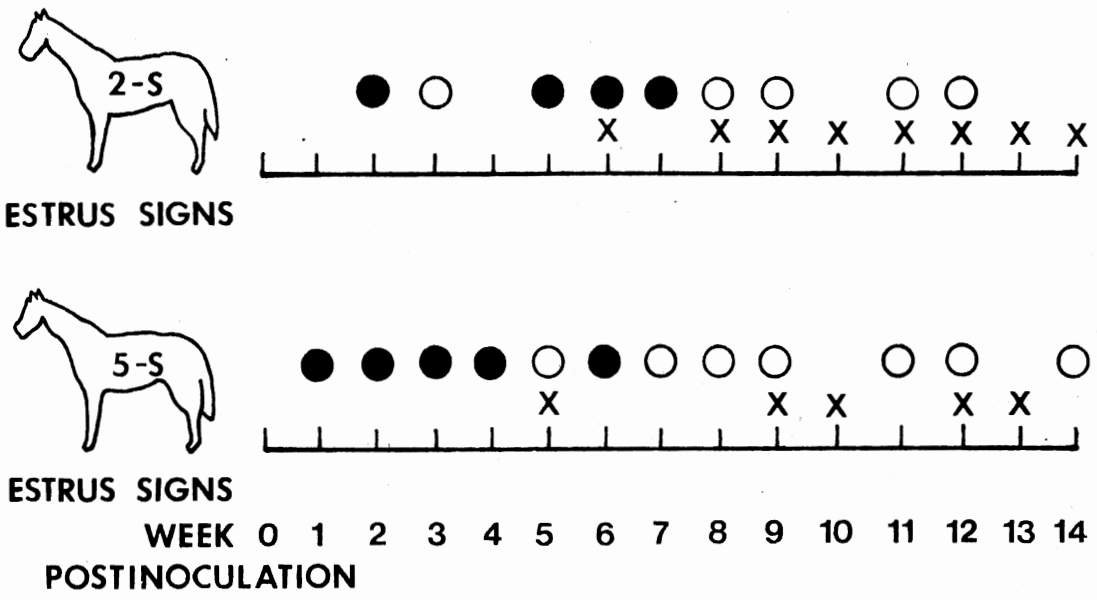
DETERMINATION OF THE NUMBERS OF K. PNEUMONIAE/ML OF UTERINE SECRETION FROM THE INOCULATED MARES IN THE PRINCIPAL STUDY

Mare	Days Postinoculation 1	7	14	21	28	35	42	FS*[1]
1	1.71×10^3	1.09×10^3	4.11×10^2	1.28×10^2	0	*[2]		0
2	3.49×10^3	0	*[3]	7.12×10^2	0	0	3.20×10^3	0
3	2.01×10^3	2.49×10^3	1.14×10^4	7.98×10^2	*[3]	2.37×10^2	2.37×10^2	0
4	6.93×10^3	5.22×10^2	2.80×10^2	5.11×10^3	5.84×10^2			0
5	2.15×10^3	3.68×10^3	3.53×10^3	1.22×10^3	1.35×10^3			0
6	3.42×10^5	5.09×10^3	5.46×10^3	7.09×10^4	2.06×10^3	1.42×10^3		0
437	2.54×10^3	1.69×10^2	6.96×10^2	3.72×10^3	*[3]	1.21×10^3	2.45×10^3	0
559	5.41×10^2	0	7.83×10^3	1.88×10^3	0			0

*[1] Final sample date, 8-30-76.

*[2] Blank spaces indicate there was no sample on this day.

*[3] Technical error; no measurement.



● *K. pneumoniae* isolated and also enumerated by plate count

○ *K. pneumoniae* isolated, but not enumerated

X Estrus signs present

Figure 1. Postinoculation Isolations of K. pneumoniae from the Uterine Specimens Obtained from Mares in the Pilot Study

from a 72 hr postinoculation uterine specimen from mare #5-S but not from mare #2-S. K. pneumoniae was isolated from the uterine specimens of mare #2-S during week 2 and thereafter until week 12 postinoculation. K. pneumoniae type 10 was isolated from the uterine specimens of mare #2-S for all but two sample dates which were 3-19-76 and 4-22-76. The organism isolated on those two dates was found to be serologically related to pool 2 (types 4, 5, 6, and 7). There were no subsequent isolations of K. pneumoniae from uterine specimens taken from this mare which includes three further uterine specimens taken in June 1976, November 1976, and January 1977. K. pneumoniae type 68 was isolated from the postinoculation uterine specimens of mare #5-S until the termination of the pilot study on May 10, 1976 which was 14 weeks postinoculation. There were no further uterine specimens taken from this mare since she was sold on termination of the pilot study. Table IX gives the results of the isolation techniques performed on the uterine specimen. The isolation of K. pneumoniae by direct inoculation of blood agar with the tampon paralleled the enumeration of the organism by the plate count method. The enrichment media inoculated with the tampon specimen was the most reliable technique for the detection of K. pneumoniae in this experiment. As each mare came out of anestrus and began to show sign of an estrus period, the numbers of K. pneumoniae isolated from the uterine specimen dropped and eventually the organism was isolated only by the enrichment technique (Tables V, VII; Figure 1).

Principal Study. The data for postinoculation isolations of K. pneumoniae from uterine specimens of the mares used in this experiment are given in Figure 2. K. pneumoniae type 68 was isolated from the preinoculation specimen from mare #1. A K. pneumoniae which was related

TABLE IX

ISOLATION OF K. PNEUMONIAE FROM UTERINE SPECIMENS OBTAINED FROM MARES IN THE PILOT STUDY

Weeks PI	Mare 2-S*[1]					Mare 5-S*[2]				
	TD	SD*[3]	PC	TE	SE*[3]	TD	SD*[3]	PC	TE	SE*[3]
0									X	
1						X		X	X	
2	X		X	X		X		X	X	X
3				X		X		X	X	
4						X		X	X	X
5	X		X	X					X	
6	X		X	X	X	X		X	X	
7			X	X					X	
8	X			X					X	
9				X					X	
10-11				X					X	
12				X					X	
13-14									X	

*[1] K. pneumoniae type 10.

*[2] K. pneumoniae type 68.

*[3] Swabs were not part of the uterine specimen from week 7-14.

PI - Weeks postinoculation; "Week 0" includes the preinoculation and 72 hr postinoculation sample.

TD - Direct agar streak with tampon cultured for K. pneumoniae.

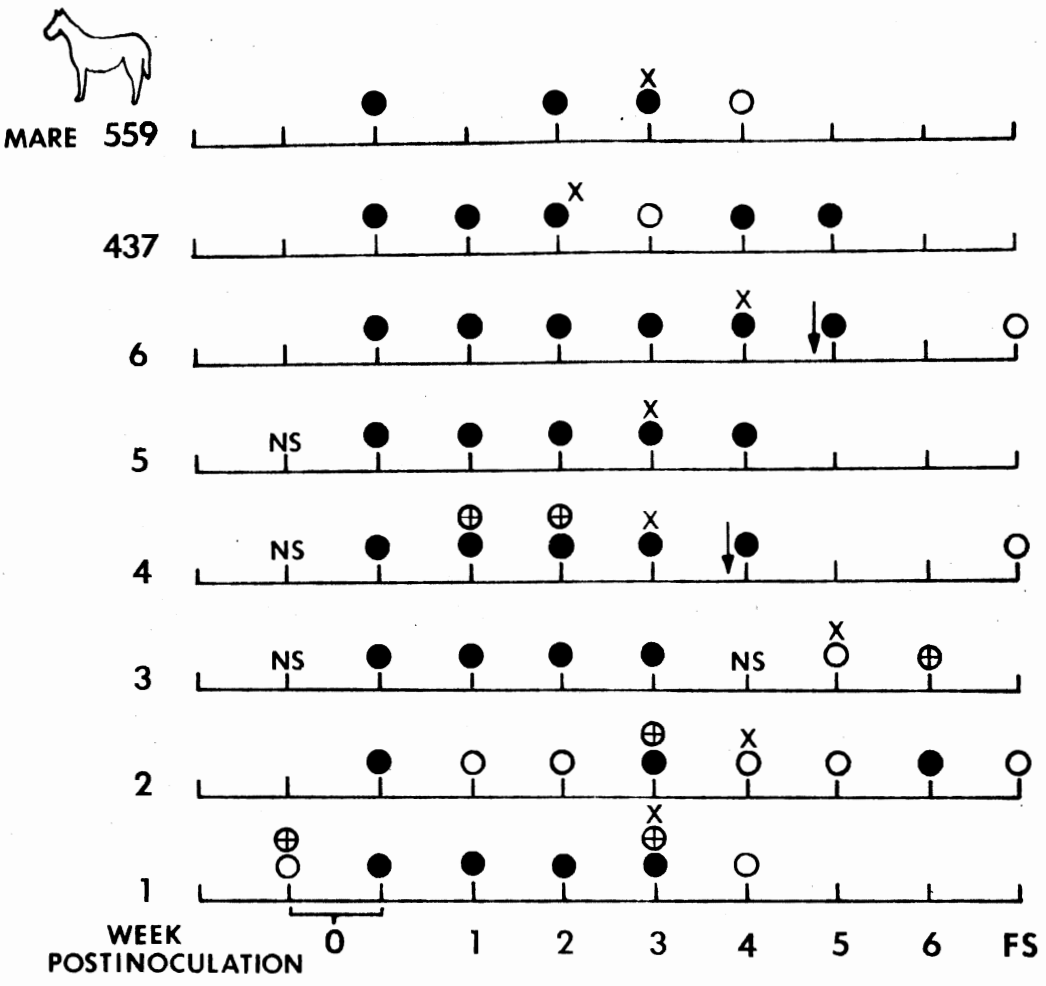
SD - Direct agar streak with swab cultured for K. pneumoniae.

PC - K. pneumoniae was enumerable by titration and plate count of of secretion.

TE - Tampon enrichment media cultured for K. pneumoniae.

SE - Swab enrichment media cultured for K. pneumoniae.

X - K. pneumoniae isolated or enumerated.



- ● *K. pneumoniae* type 68 isolated and also enumerated by plate count
- ○ *K. pneumoniae* type 68 isolated, but not enumerable
- ↓ The number of *K. pneumoniae* enumerated decreased from last sample
- X Estrus period onset
- ⊕ *K. pneumoniae* isolated but not of type 68
- NS No sample obtained due to technical error
- FS The final sample date (8-30-76), see table I. for the number of weeks of postinoculation this sample represents for each mare.

Figure 2. Postinoculation Isolations of K. pneumoniae from the Uterine Specimens Obtained from the Inoculated Mares in the Principal Study

to types 22 and 23 was also isolated from this uterine specimen (Table XI). Mare #1 had been previously sampled one month earlier and no K. pneumoniae were isolated from that uterine specimen. A K. pneumoniae which was found to be in the pool containing capsule types 4, 5, 6, and 7 was isolated from a uterine specimen taken from mare #437 nine days before inoculation. The organism was never again isolated from mare #437 or any other mares in the experiment. The 24 hr post-inoculation specimens from the eight mares inoculated with K. pneumoniae type 68 yielded the organism. K. pneumoniae type 68 was isolated from all of the uterine specimens of the infected mares through the sample period which lasted for at least one estrous cycle. The numbers of K. pneumoniae isolated from the uterine specimens of seven of the eight mares dropped after the mares completed the postinoculation estrous cycle (Table VIII; Figure 2). On the final sample date (8-30-76), K. pneumoniae type 68 was isolated by the enrichment technique from the uterine specimen of three of the eight infected mares. Table X gives the data for the isolation of K. pneumoniae cultured from the uterine specimens. The isolation of K. pneumoniae by direct inoculation of blood agar with the tampon paralleled the enumeration of the organism by the plate count method.

A K. pneumoniae related to capsule type 22 and type 23 was recovered from uterine specimens of four of the inoculated mares (Table XI). There were no other isolations of this organism from the subsequent uterine specimens from these mares. Another serotype belonging to pool 16 (types 60, 61, 62, 63) was isolated from mare #3 from the specimen obtained 6 weeks postinoculation.

TABLE X

ISOLATION OF *K. PNEUMONIAE* TYPE 68 FROM UTERINE SPECIMENS OBTAINED FROM MARES IN THE PRINCIPAL STUDY

Day*[1]	TD	PC	TE	Day	TD	PC	TE	Day	TD	PC	TE	Day	TD	PC	TE
	Mare 1				Mare 2				Mare 3				Mare 4		
0	X	*[2]	X	0				0				0			
1	X	X	X	1	X	X	X	1	X	X	X	1	X	X	X
7		X	X	7			X	7	X	X	X	7	X	X	X
14	X	X	X	14	X		X	14	X	X	X	14	X	X	X
21	X	X	X	21	X	X	X	21	X	X	X	21	X	X	X
28	X		X	28			X	28*[3]				28		X	X
				35			X	35		X	X				
				42	X	X		42			X				
70				98			X	84				70			X
	Mare 5				Mare 6				Mare 437				Mare 559		
0				0				0				0			
1	X	X	X	1	X	X	X	1	X	X	X	1	X	X	
7	X	X	X	7	X	X	X	7	X	X	X	7			
14	X	X	X	14	X	X		14	X	X	X	14	X	X	X
21	X	X	X	21	X	X	X	21	X	X	X	21		X	X
28	X	X	X	28	X	X	X	28	X		X	28	X		X
				35	X	X	X	35	X	X	X				
								42	X	X	X				
70				49	X		X	98				70			

*[1] Number of days postinoculation; "Day 0" is the day of inoculation.

*[2] Blank spaces indicate *K. pneumoniae* not recovered from the specimen.

*[3] No specimen taken on this day.

TD - Direct agar streak with tampon cultured for *K. pneumoniae*.

PC - *K. pneumoniae* was enumerable by titration and plate count of secretion.

TE - Tampon enrichment media cultured for *K. pneumoniae*.

X - *K. pneumoniae* was isolated or enumerated.

TABLE XI

ISOLATION OF A K. PNEUMONIAE RELATED TO CAPSULE TYPES 22 AND 23 FROM UTERINE SPECIMENS OF FOUR MARES IN THE PRINCIPAL STUDY

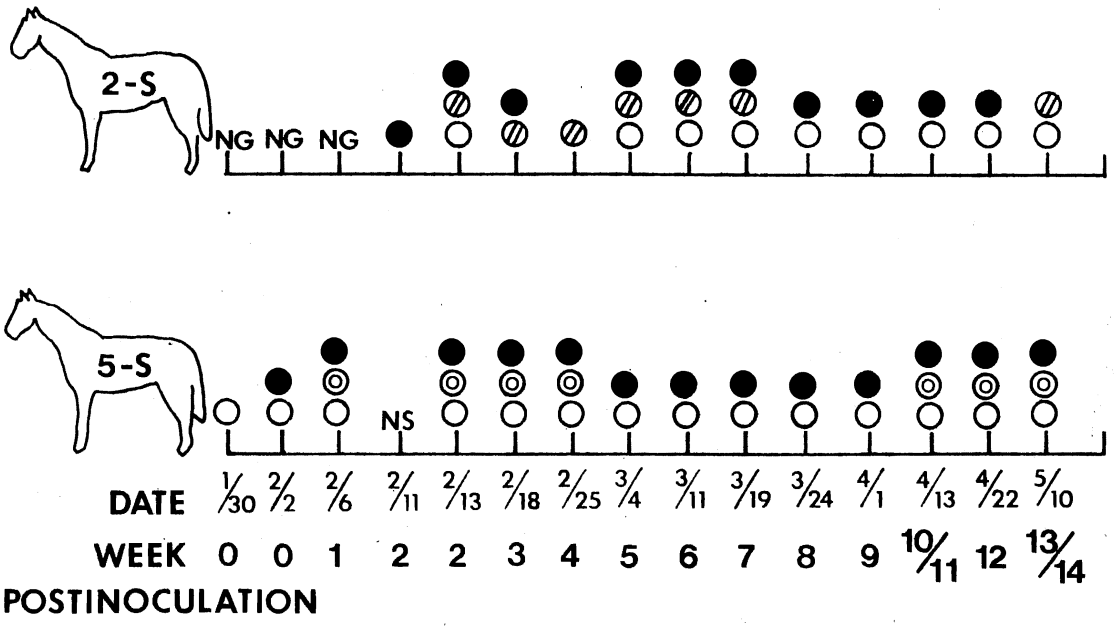
Date	Mare	Week PI
6/11/76	#2, #4	3, 1
6/18/76	#1, #4	0, 2
7/09/76	#1	3

K. pneumoniae was not recovered from the uterine specimens of the control mares taken before or after inoculation of the uterus with sterile Worfel-Ferguson broth.

Comprehensive Isolations of Bacteria From the Uterine Specimens of Mares in This Study

Pilot Study. There were no bacteria isolated from the preinoculation specimen obtained from mare #2-S. The preinoculation specimen of mare #5-S yielded E. coli.

Bacteria other than K. pneumoniae that were regularly isolated from the postinoculation uterine specimens were E. coli, Pseudomonas spp. and Streptococcus spp. The Streptococcus spp. isolated were found to belong in the Lancefield group C and were identified as S. zooepidemicus, S. equisimilis, and S. canis (Figure 3).



● *K. pneumoniae* isolated

○ *E. coli* isolated

◎ *Streptococcus* sp. isolated

⊘ *Pseudomonas* sp. isolated

NG No bacterial growth from culture of specimen

NS No uterine specimen was obtained on this day

Figure 3. Comprehensive Postinoculation Isolations of Bacteria from the Uterine Specimens Obtained from Mares in the Pilot Study

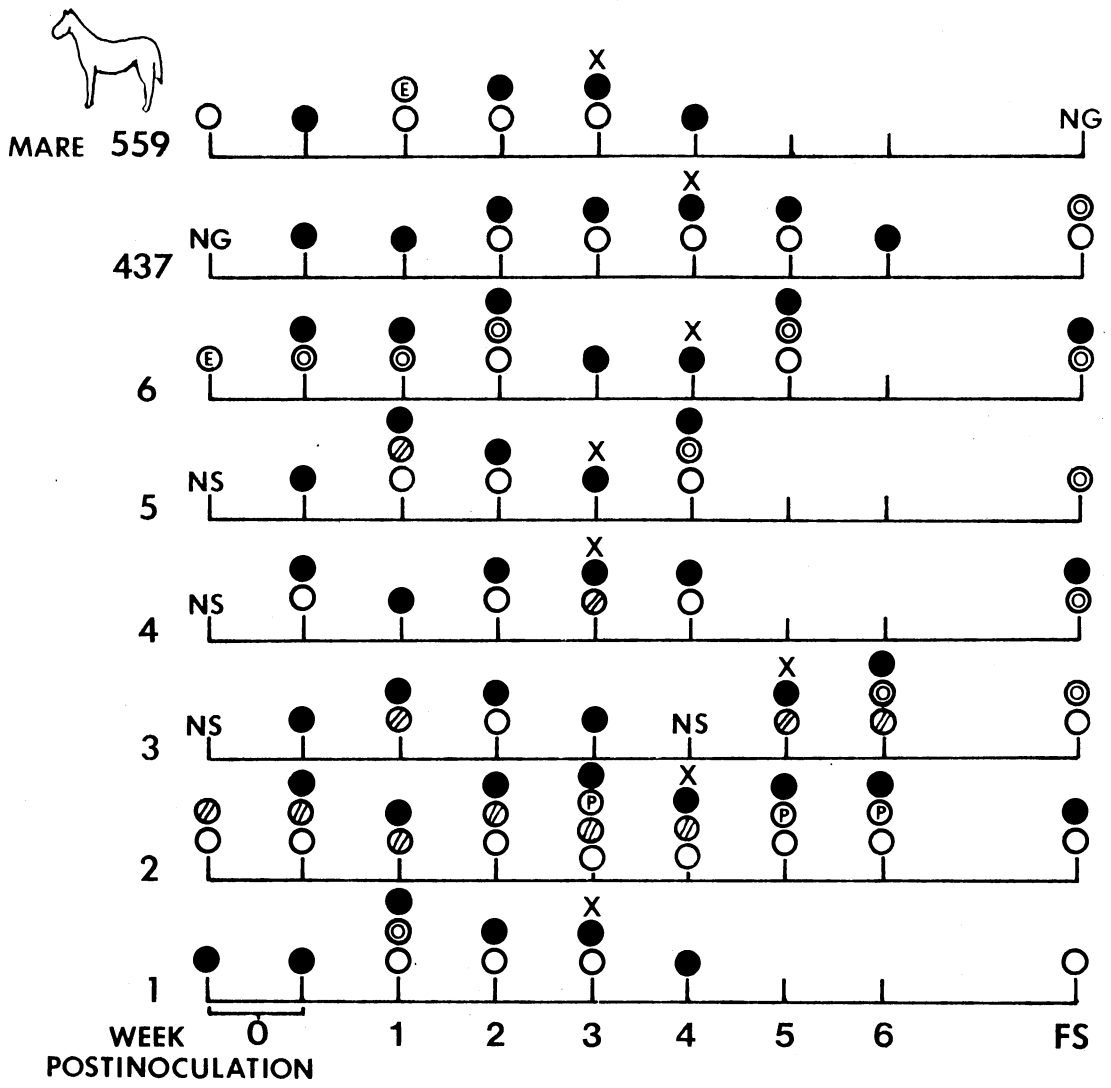
Principal Study. The preinfection specimens of the mares yielded E. coli in all instances. One specimen from #5 yielded an Arizona sp. and one specimen from #4 yielded both an Enterobacter sp. and an Alcaligines sp. Two specimens yielded K. pneumoniae as aforementioned.

Bacteria other than K. pneumoniae that were regularly isolated from the postinoculation uterine specimens were E. coli, Proteus vulgaris, Pseudomonas spp., and Streptococcus spp. (Figure 4). The Streptococcus spp. isolated were found to belong in the Lancefield group C and were identified as S. canis and S. zooepidemicus.

Organisms regularly isolated from the postinoculation uterine specimens of the control mares were E. coli and Streptococcus zooepidemicus (Figure 5). There was no K. pneumoniae isolated from the uterine specimens of the control mares.

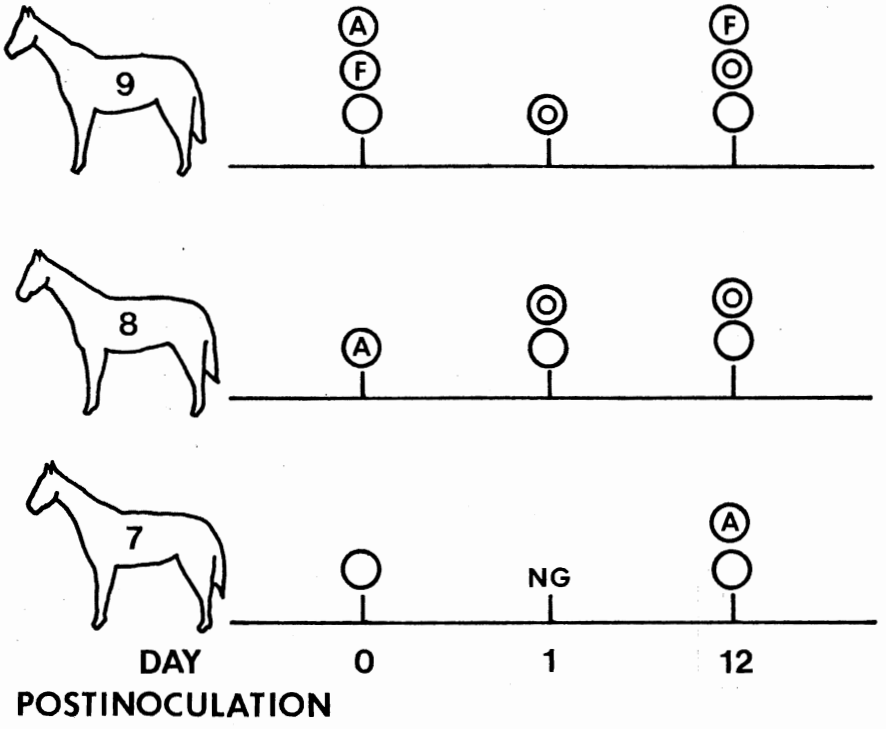
Examination of Frozen Sections of Biopsied Uterine Tissue Using a Fluorescent Antibody Staining Technique

The conjugate to be used for the staining of the fixed frozen sections was tested using smears of K. pneumoniae type 68, type 30, and a K. pneumoniae which was a serotype in pool 16 (60, 61, 62, and 63). A positive reaction of K. pneumoniae type 68 with the conjugate consisted of capsule swelling of the organism giving it the appearance of a fluorescing donut. When the heterologous capsule types were stained with the conjugate, no capsule swelling occurred but the cell wall did fluoresce. The conjugate caused capsule swelling with 4+ fluorescence at a dilution of 1:8 until it had been frozen at -70°C and then the capsule swelling with fluorescence did not occur at a dilution greater than 1:2.



● *K. pneumoniae* isolated
○ *E. coli* isolated
◎ *Streptococcus* sp. isolated
⊘ *Pseudomonas* sp. isolated
Ⓔ *Enterobacter* sp. isolated
Ⓟ *Proteus vulgaris* isolated
NG No bacterial growth from culture of specimen
NS No uterine specimen was obtained on this day
X Estrus period onset
FS Final sample date (8-30-76)

Figure 4. Comprehensive Postinoculation Isolations of Bacteria from the Uterine Specimens Obtained from Inoculated Mares in the Principal Study



○ *E. coli* isolated

◎ *Streptococcus* sp. isolated

Ⓐ Actinomycetales isolated

Ⓕ Fecal Streptococcus isolated

NG No bacterial growth from culture of specimen

Figure 5. Isolations of Bacteria from the Uterine Specimens Obtained from Uninoculated Mares in the Principal Study

Pilot Study. Seventeen of the twenty-three biopsy samples were successfully stained and examined. The sections from the first six were damaged during the staining process and could not be examined. There were no K. pneumoniae observed in any of the seventeen specimens observed. Small groups of ovoid fluorescing bodies were observed in the interglandular endometrium of the sections. Smears of K. pneumoniae type 68 stained with the conjugate served as controls, and when the size of the ovoid bodies found in the stained sections were compared to the controls, the ovoid bodies were found to be smaller than K. pneumoniae. The fluorescence of the small bodies was not eliminated or reduced by prior treatment of the sections with non-conjugated antibody to K. pneumoniae type 68.

Principal Study. No K. pneumoniae were observed, however the ovoid fluorescing bodies were also found in the interglandular endometrium. The two sections of each specimen which were stained with FITC conjugated antisera to Clostridium oediamatens also showed the fluorescing small ovoid bodies.

Serologic Techniques

Detection of Serum Antibody to K. pneumoniae From Samples Obtained from Mares in This Study

Pilot Study. Three serotypes of K. pneumoniae isolated from the uterine specimens of the pilot study mares were used to test serum taken from the mares on 6-4-76 for presence of capsule swelling antibody. K. pneumoniae type 68, type 10, and a K. pneumoniae in pool 2 isolated from

postinoculation uterine specimens of mare #5-S, #2-S, and #2-S respectively were used for the test. The serum from both mares elicited a capsule swelling of K. pneumoniae type 68 and the K. pneumoniae in pool 2, however, it did not elicit capsule swelling of K. pneumoniae type 10.

Principal Study. Sera obtained from the mares in this study inoculated intrauterinely with K. pneumoniae type 68 were tested for presence of capsule swelling antibody to K. pneumoniae type 68 (Table XII). All mares either had capsule swelling antibody to the organism prior to intrauterine inoculation or developed the antibody after intrauterine inoculation of the organism. No titers greater than 1:8 were detected. None of the sera taken from the controls on any sample date elicited capsular swelling of K. pneumoniae type 68.

Production of Capsular Antisera to K. pneumoniae Serotypes 2, 5, and 7

The sera from the rabbits were tested for antibody to K. pneumoniae serotypes 2, 5, and 7 by the capsular swelling technique and a slide agglutination technique. Table XIII gives these data. Antibody to serotypes 2 and 7 was detected by both techniques. Antibody to serotype 5 was not detectable.

TABLE XII

SERA TITERS*[1] TO K. PNEUMONIAE TYPE 68 AS DETECTED BY THE CAPSULE SWELLING TEST USING SAMPLES OBTAINED FROM INFECTED MARES IN THE PRINCIPAL STUDY

Date	Mare 1	Mare 2	Mare 3	Mare 4	Mare 5	Mare 6	Mare 437	Mare 559
6-04	*[2]	+					-	
6-11	+	1:2	-	1:4	-	1:4	-	-
6-18	1:2	1:2	1:2	1:4	-		+	1:2
6-24	1:2	1:2	1:2	1:4	+	+	1:2	+
7-02	1:2	1:2		1:4	1:2		1:2	1:2
7-08						1:2		
7-09	1:4		1:2			1:2		1:2
7-16	+		-			1:4		+
7-23						1:2		
7-30						1:8		
8-05						1:4		
8-16						1:2		
8-30	1:2	1:4	-	1:2	+	1:4	-	1:2

*[1] Titers listed are highest dilution of sera from which capsule swelling of K. pneumoniae type 68 was observed.

*[2] Blank space indicates no test of serum on this date.

+ Capsule swelling of K. pneumoniae type 68 was observed with undiluted serum.

- No capsule swelling of K. pneumoniae type 68 was observed.

TABLE XIII

ANTISERA TITERS PRODUCED TO WHOLE CELL ANTIGEN OF K. PNEUMONIAE SEROTYPES 2, 5, AND 7

Titer	Rabbit No.	Capsule Type Antigen 2	5	7
Slide Agglutination Titers	10*[1]	1:20*[4]	-	-
	11*[2]	-	-	-
	23*[3]	-	-	1:10
Capsule Swelling Titers	10	1:16	-	-
	11	-	-	-
	23	-	-	1:8

*[1] Inoculated with K. pneumoniae type 2.

*[2] Inoculated with K. pneumoniae type 5.

*[3] Inoculated with K. pneumoniae type 7.

*[4] Titers listed are the high dilution of sera from which agglutination or capsule swelling was observed.

CHAPTER V

DISCUSSION

Use of a Tampon as a Uterine Sampling Device

The standard method for taking uterine samples for bacterial isolation using guarded culture swabs was found to offer a very small surface area for contact with the area sampled. The implementation of tampons for obtaining uterine specimens served to (1) increase the surface area sampled, (2) increase the amount of uterine secretion collected, and (3) increase the time of contact of the device with the area sampled. The use of tampons for the absorption of secretions from mucous membranes in cattle was also a basis for this choice of a sampling device[25]. The tampon method of obtaining the uterine specimen was better economically than the more expensive guarded culture swabs. One box of sixteen tampons usually cost less than one guarded culture swab, and the tampons were available in most grocery stores.

The problem of inserting the tampon into the cervix of a mare as cleanly as possible to avoid contamination from the vaginal canal posed a problem, and that was why Method A was originally developed. Method A was found to be inconvenient, time-consuming, and therefore Method B was devised. When the later routine sampling was done carefully with Method B, it was possible to get bacterial free samples from an uninfected mare. However, for field studies Method B may be impractical for those mares with surgical closures of the vagina (Caslick) and Method A or the

guarded swab might be substituted for those cases. When using Method A two specula must be used to prevent the cervix and vagina from being exposed to the air for ten minutes. If the mare evacuated during this ten minutes and the use of only one speculum was attempted, there would be a greater risk of contamination from excreta.

When comparing the efficacy of recovery of the organism with the guarded swab to that with the tampon, results of this study (Table IV) gave an indication that the tampon was a more efficient way to recover K. pneumoniae from the uterus that the swab. The use of sanitary tampons was employed in this study to possibly increase the rate of isolation of K. pneumoniae from the uterus. It was found that of the fourteen samples compared, not once was K. pneumoniae recovered by direct streak of blood agar with a swab, while nine tampon specimens showed K. pneumoniae on the direct agar streak. In comparing the enrichment recoveries, the tampon specimens showed K. pneumoniae in eleven, while the swab specimens only showed K. pneumoniae in three. The tampon surface was, of course, greater and a larger sample of the uterine mucous membrane and secretion was obtained with the tampon than with the guarded swab. Recalling that this infection has been reported to be confined to the mucous membrane of the genital tract[12, 13], this factor of the difference in surface area between the tampon and swab may be of considerable importance in isolating the organism.

Disinfection of Contaminated Biopsy Forceps

The 1:40 chlorhexidine used for disinfection of the forceps in the pilot study proved unsatisfactory. It was thought that this disinfectant would be efficacious against K. pneumoniae when coupled with a

thorough scrubbing of the contaminated forceps and rinse with sterile saline; as given in the results, K. pneumoniae was not eliminated from the forceps using this procedure. The forceps were soaked in the 1:40 chlorhexidine between each sample and therefore they were in contact with the disinfectant for at least five minutes. The time requirement for disinfection of the forceps using chlorhexidine was evidently longer, and rather than risk possible transfer of organisms between mares a more efficacious disinfection procedure was sought. A disinfection procedure using 70 percent isopropyl alcohol volume per volume was considered for three reasons. First, it has been shown to be efficacious in a short time exposure (less than 10 minutes) against E. coli and Streptococcus[10, 32]. These were bacteria known to be isolated from the uterine specimens in the pilot study (Figure 3). Second, 70 percent was found to be the optimal concentration necessary[10, 32]. Third, there was no difference found between the efficacy of isopropyl alcohol and ethyl alcohol[10, 32], therefore isopropyl was chosen because it is less expensive. By using a volume of 1 liter of alcohol to disinfect the forceps, the possibility of any residual saline from the rinse of the forceps diluting the alcohol was eliminated.

Selection of Organism for Inoculation

A comprehensive survey to compile data for the most prevalent capsule type of K. pneumoniae was found to be impossible due to lack of cooperation from breeding farms. The eight isolates of K. pneumoniae which were submitted to this researcher for capsule typing were isolated during the fall of 1975. Six of these eight isolates were found to be capsule type 68, one was found to be capsule type 10, and one was found

to be capsule type 30. Several isolates of K. pneumoniae which were isolated prior to the fall of 1975 were found by the CDC, Atlanta, GA, to be capsule types 68 and 30[37]. The K. pneumoniae type 68 was chosen for the inocula because it was the serotype most commonly isolated from stallions and therefore it was assumed mares were most likely to come into contact with it. The K. pneumoniae type 10 was chosen because it was the least commonly isolated serotype.

Quantitation of Uterine Secretions as a Means by Which to Experimentally Monitor Infection In the Uterus

Quantitation of the amount of uterine fluid collected from the uterine specimen and counts of bacteria in the fluid gave data for two methods of monitoring the infection in the uterus. First, by correlating the numbers of K. pneumoniae/ml of uterine fluid and the estrous cycles of the mares, a relationship was established. In both the pilot study and the principal study there was a pattern of a decrease in the numbers of K. pneumoniae/ml uterine fluid after the mares completed a postinoculation estrous cycle. Second, the amount of uterine fluid apparently increased 24 hr postinoculation of the uterus with K. pneumoniae. Histological examinations performed on biopsies taken from the 24 hr postinoculation uterine specimens found an acute inflammatory response occurring in the endometrium at this time[18]. One mare #437, showed an increase of uterine fluid again at week five postinoculation. This second increase was concurrent with an apparent exacerbation of the acute inflammatory response determined by histological examination of the uterine biopsy taken at that time[18].

Effect of the Estrous Cycle on Isolations of *K. pneumoniae* from the Uterine Specimen

In both the pilot study and the principal study the onset of an estrous cycle affected the isolation of *K. pneumoniae* from the postinoculation uterine specimen (Tables V, VII, VIII; Figures 1 and 2). In all mares but one, #5, the numbers of *K. pneumoniae*/ml uterine fluid dropped with the onset of the estrous cycle following inoculation. In five mares the organism was isolated only by use of the enrichment medium after the onset of an estrous cycle and *K. pneumoniae* were not enumerable in the secretions. These data demonstrate the efficacy of a host non-specific defense in controlling an infection. Merkt, *et al*, made specific reference to this attribute of mares with an infected uterus to clear the infection without antibiotic treatment[26].

Identification of *K. pneumoniae* Isolated From Uterine Specimens of Mares in This Study

The mucoid glistening colonies of *K. pneumoniae* on blood agar medium were quite characteristic after 24 hr incubation at 37°C unless they were crowded or overgrown. Colonies of non-motile *E. coli* and encapsulated *Enterobacter* strains could be mistaken for *K. pneumoniae*, however the reactions on TSI slants, Simmons citrate slants, SIM, urea slants and the tests (anerobic) for lysine decarboxylase, ornithine decarboxylase, and arginine dehydrolase will sufficiently differentiate *K. pneumoniae* from these other two genera[7]. The criterion of motility has been reported as especially important for differentiating some *Enterobacter* spp. from other *Klebsiella* spp.[6], but no difficulties differentiating these two were encountered in this study, even though

on occasion this researcher was given an Enterobacter sp. to be typed which had been mistaken by someone else for a Klebsiella sp.

In both experiments there were K. pneumoniae serotypes isolated from the uterine specimens which were not inoculated into the uterus. In this study it was not determined whether the capsular antigenic composition of the organism used for inoculation was fluctuating, or if new serotypes were introduced into the uterine specimens from the environment and coexisting with the serotype inoculated. Merkt, et al, reported a similar circumstance in which a mare was found to have a uterine infection with K. pneumoniae type 5 and subsequent cultures of uterine samples yielded K. pneumoniae type 7[26].

In the pilot study on two occasions the K. pneumoniae isolated from mare #2-S would cross react with the polyvalent antisera of both pools 2 and 3. Pool 3 contains antisera to capsule types 8, 9, 10, and 25. Pool 2 contains antisera to capsule types 4, 5, 6, and 7. The specific type antisera included in pool 3 were on hand in the laboratory whereas the only specific type antisera on hand in the laboratory included in pool 2 was type 7 which was produced in rabbits by the researcher. Type 8, 9, 10, and 25 antisera would not cause capsule swelling of this isolate, and the type 7 antisera would cause capsule swelling of this isolate. The type 7 would also occasionally cause capsule swelling of type 10 cells used for the inoculation of mare #2-S. Types 10 and 7 are known to occasionally cross react[3]. It was not possible to determine exactly what was happening but there may be two possibilities. One, the antigenic structure of the capsule may occasionally change slightly and since types 7 and 10 are known to cross react, the differences in the antigen structure may be very slight to begin with. Second, the mare

may have incurred a second infection during the course of the experiment with a type 7 organism, the source of which is unknown at this time.

In the principal study, K. pneumoniae which were not type 68 were isolated from four mares (Table XI). The colonies of these isolates on blood agar medium were smaller than K. pneumoniae type 68; on TSI they were not as mucoid nor did they produce an alkaline slant in 3 days as was usual for the type 68 strain; and they failed to produce gas from glucose fermentation broth. After first isolating this strain from mares #2 and #4 on 6-11-76, it was later isolated from mares #1 and #5 (Table XI). K. pneumoniae type 68 were also isolated from the uterine specimens of the four mares on the same dates. This researcher was not able to type the capsule of this strain of K. pneumoniae and it was sent to the CDC, Atlanta, GA, for typing. It was found to be a K. pneumoniae related to types 22 and 23^5. When this organism was observed in a smear stained with nigrosin, it was found to quite pleomorphic and the cells had capsules much smaller than those of the type 68 strain.

A K. pneumoniae belonging to pool 16 (types 60, 21, 62, and 63) was isolated from the uterine specimen of mare #3 of the principal study on July 16 (6 weeks postinoculation) from the methyl violet enrichment medium. It did not differ from type 68 biochemically or morphologically. No K. pneumoniae type 68 were isolated from this specimen. The pool 16 strain did not cross react with the type 68 antisera. Sera taken from the mare on July 16 did not cause capsule swelling of the pool 16 strain cells or the type 68 strain cells, however sera from two previous dates had caused capsule swelling of the type 68 strain cells (Table XII). There were no subsequent isolations of either pool 16 or type 68 strains from the last uterine specimen taken from this mare. The source of the pool 16 strain was not determined.

Postinoculation Isolations of Bacteria From the Uterine Specimens

In both experiments the postinoculation samples showed multiple infections evidenced by a variation of organisms isolated from the uterine sample (Figure 3, 4). No direct correlation between the numbers of *K. pneumoniae* present and the effect of the presence of other organisms was found from this study. Isolation of a *Pseudomonas* sp. was recurrent in two mares and *Streptococcus zooepidemicus* and *S. canis* were recurrent in two mares. *E. coli* which were non-hemolytic, was the most commonly isolated organism besides *K. pneumoniae*.

Comparing the isolations from the controls to those from the principals there was evidence that the mere act of sampling the horses can possibly introduce organisms into the genital tract with subsequent colonization (Figure 5). It has been suggested that the isolation of organisms, especially *Streptococcus* spp. and *E. coli*, from uterine samples is not necessarily a reflection of a uterine infection, but may be organisms colonizing the vagina[28, 33].

Accidental Transmission of *K. pneumoniae* Type 68 to One Experimental Mare

A *K. pneumoniae* type 68 was isolated from the preinoculation specimen obtained from mare #1 on June 18 (Figure 4). Mare #1 was sampled previously on May 10 along with the two pilot study mares and no *Klebsiella* spp. were isolated from her. Uninfected mares were always sampled before the infected mares as a standard practice.

The experiment to evaluate disinfection of the biopsy forceps using 1:40 chlorhexidine showed that the biopsy forceps were not adequately

disinfected by that method. The 70 percent disinfection method was not yet being used on May 10. However, that fact does not make any difference here since mare #1 was sampled before the infected mares and the forceps were always autoclaved between sampling periods. Other possible exposures to the organism include the following: (1) the wash water used during examination, (2) mechanical transfer by the tease stallion, and (3) daily rectal palpations of the reproductive tract performed by a group of Veterinary students using these mares in an elective course.

The wash water during examination could have become contaminated while the mares were being prepared since the mares were washed simultaneously. The procedure of using a new plastic liner in and fresh water for the buckets used for sampling each mare was not implemented until the principal study mares were moved to the Pawhuska research station. In the later experiment, care was taken to never contaminate the water with the hand used for washing the mare, by using one hand to wash and the other only for getting the wet cotton from the wash bucket and two mares were never washed with the same water.

One month before this mare was found to be infected, nasal specimens were cultured and showed no K. pneumoniae. Retrospectively, the possibility that the tease stallion mechanically transferred the organism during teasing from an infected pilot study mare to the #1 mare by previous contact of the stallions muzzle with the vulva of a pilot study mare should not be overlooked.

The students performing the daily rectal exams before this mare was moved to Pawhuska were instructed not to introduce any contaminated speculum or rectal glove into the vagina of the mares. Assuming that the students followed these instructions, the possibility of infecting this mare by those students may be ruled out.

The exact means by which this mare became infected before the introduction of the experimental inoculum to this point is unknown.

Capsule Swelling Reaction

A number of trial tests using organisms of known capsule types were necessary not only to familiarize oneself to the capsule swelling test but also to find the proper working dilutions. When using the Difco commercial antisera and following that set of instructions, it was difficult at first to obtain a positive test. The following changes were made to obtain successful results:

1. The pooled antisera used in this study were not diluted as per instructions when first typing an organism. Some of the pools seem to give very week positives unless used full strength, and other researchers have found it necessary to supplement some pools with specific type sera to obtain positive capsule swelling[10, 36, 35]. Pools were not supplemented in this study. When two or more pools caused capsule swelling of an isolate, then a twofold dilution series to 1:16 of those pools were necessary to dilute out the cross reaction. The suggested working dilution by Difco of 1:8 - 1:16 was a procedure of questionable reliability but undiluted antisera was found to be reliable.
2. A dye such as methyene blue was not necessary to see the capsule swelling, and it caused agglutination of particles in the serum. Nigrosin was used by Casewell[10] but it was found in this study that unless the dye was diluted 1:1000, the organism would autoagglutinate in the serum creating a

masking effect of the true agglutination of the cells caused by the antisera and mask the capsule swelling reaction.

3. The use of Worfel-Ferguson agar in plastic petri dishes to grow the bacteria to be typed was used in lieu of broth culture. With practice, this researcher was able to suspend the right amount of bacteria from the plate into 3-5 ml of phosphate buffered saline to attain microscope fields with 10-25 organisms per field (970X). By using agar media in petri dishes it was easy to tell if the culture was pure by typing more than one individual colony. Casewell[10] used a modified tissue grinder to homogenize the bacterial suspension used for the capsule swelling test, but in this study it was found that a vortex mixer worked adequately for mixing the suspension in the tube if the suspensions were allowed to stand 30 minutes after mixing, giving the clumps time to dissociate. The suspension for the capsule swelling test was usually correct when the phosphate buffered saline just began to get cloudy.

The capsule swelling test was a simple test to perform but the results were variable and difficult to interpret when typing strains with small capsules. The nature of the capsule is, of course, of utmost importance to the success of the test[5, 10, 20, 21]. It was found that the correct recognition of a positive capsule swelling reaction requires a certain degree of experience especially when trying to interpret weak reactions. The organism to be typed must first be identified biochemically as a Klebsiella sp., as it has been reported that certain encapsulated E. coli have identical capsules to certain Klebsiella spp. capsule types[14, 21]; therefore a positive capsule swelling of an organism

with Klebsiella capsule antiserum does not necessarily mean the organism is a Klebsiella sp.

Fluorescent Antibody Technique

The conjugation of rabbit antisera to K. pneumoniae type 68 was successful but the following problems were encountered:

1. Freezing the conjugate at -70°C partially destroyed the capsule swelling ability of the antibody. The conjugate gave bright (4+) fluorescence at a dilution of 1:8 before freezing. When aliquots of the same conjugate had been frozen for 10 days, and subsequently thawed and used for staining of smears of K. pneumoniae type 68 cells, it gave only moderate fluorescence (3+) at a maximum dilution of 1:2. It was observed that as the aliquots were thawed they became cloudy, and on the first thawing of an aliquot this precipitate was spun out. When a new aliquot was tried, this precipitate was spun out but allowed to go back into solution by letting the aliquot stand at refrigerator temperature for 3 days. There was no difference in the titers between the first and second aliquots.
2. A background fluorescence was very evident in the stained sections of the uterine biopsies. The use of 20 percent chicken tissue homogenate[11] was helpful in reducing the background fluorescence somewhat but did not eliminate it.

The conjugated antibody was specific for K. pneumoniae type 68. It did not stain capsules of type 30 or any of the pool 16 serotypes. Because the conjugate worked successfully on smears, it was assumed that when mixed with 20 percent chicken tissue suspension in a 1:2 dilution

(to reduce the non-specific staining) the conjugated antibody would produce sufficient staining to identify type 68 cells in the stained frozen sections.

The fact that the organism was present in low numbers ($< 10^4$/ml) may account for not observing it in the stained frozen sections.

The difference in the morphology of the K. pneumoniae type 68 in smears stained with the antibody conjugate was sufficiently different from that of a K. pneumoniae type 30 stained with the same conjugate to undoubtedly identify the positive result. When an aberrant reaction occurred, the organisms were shown to have capsules smaller than normal and the smears were found to have been made from a culture older than 48 hr.

Capsule Swelling of K. pneumoniae Type 68 By Sera From Mares in This Study

Crouch, et al, reported that the sera collected post mortum from the infected stallion did not cause capsule swelling of K. pneumoniae type 5 isolated from the stallion[12]. All sera from the infected mares in this study caused capsule swelling of K. pneumoniae type 68 cells. The noninfected mares sera did not cause capsule swelling of type 68 cells and this gives some indication that the low titer in the infected mares sera may have been due to the intrauterine infection with K. pneumoniae type 68. Perhaps a consideration to the biopsy itself should be given when trying to interpret these results. In the natural infection, the weekly biopsy puncture of the endometrium would not be present, and an inflammatory response would probably not be normally induced unless there was an abortion or parturition. Another explanation could

be that the capsule swelling observed was the result of antibody present in the sera to other bacteria which have capsular antigens identical to K. pneumoniae type 68.

Comment on the Uterine Infection Produced in Mares in This Study

The criterion for production of the infection was that of subsequent reisolations of the organism from the uterine specimens of the inoculated mares. The catarrh often associated with infections with K. pneumoniae was evident in only three of the ten mares inoculated and it was slight in those cases. There is the possibility that an artificial uterine carrier state was produced in some of these mares. If after the estrous cycle, the organisms had been cleared from the uterus and had then localized in the urethra or clitoral area, the culture of the organism from the tampon may have then actually originated from contamination of the vaginal canal from the external genitalia or urethra. Since the external genitalia and urethra were not sampled in this study that question remains to be answered.

It was not determined in this study if the K. pneumoniae used for inoculation would actually cause infertility in the mare. The organism used for inoculation was originally isolated from stallions with a history of breeding problems and therefore was assumed to be infective for the stallion. This emphasizes the importance of the infection produced in the mare because of the proved venereal transmission of this organism.

Summary

Two experiments incorporating thirteen mares were used to collect the data used in this thesis, the pilot study and the principal study. The purpose of the experiments was to produce and to monitor the intrauterine infection with K. pneumoniae. In the pilot study the infection was produced with K. pneumoniae type 68 and type 10. In the principal study K. pneumoniae type 68 was used to produce the infection. These strains of K. pneumoniae were isolated from the genital tract of stallions with a history of breeding problems. Tampons and guarded culture swabs were used to obtain uterine specimens in the pilot study. The efficacy of the isolation of K. pneumoniae using the tampons was compared to that with the use of the standard guarded culture swab, and the tampon proved to be a more reliable means with which to isolate K. pneumoniae and was used in the principal study. In both studies, inoculated mares became infected and remained infected at least until the postinoculation estrous cycle was initiated or was completed. Some of the inoculated mares remained infected through more than one estrous cycle. The numbers of K. pneumoniae decreased in the uterus of all mares after completing the estrous cycle following inoculation. K. pneumoniae was not demonstrable in tissue sections of the uterine specimens stained by the fluorescent antibody technique. Postinoculation sera antibody titers to K. pneumoniae as determined using the capsule swelling technique were found to be no higher than 1:8.

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VITA 2

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