The effects of environmental fomites on the survival of pseudorabies virus

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

GENERAL INTRODUCTION AND LITERATURE REVIEW

This thesis deals with the effects of environmental fomites, fresh animal waste products and animal wastes in waste handling systems on the survival of pseudorabies virus (PRV) in the environment.

PRV, classified as a porcine herpesvirus, Herpesvirus suis, is composed of a DNA core surrounded by an icosahedral capsid, completely enclosed by a lipoprotein envelope.[24] The virus is capable of causing clinical disease in all domestic livestock and in a wide range of wild animals[17,19] with symptoms ranging from those of respiratory disease and reproductive problems, to intense pruritus, followed by neurological disorders and eventual death.[27] Swine, the primary hosts for the organism,[19] display different clinical signs depending upon their ages when infected, the strains of the virus involved and management of the herd.[20,27] Piglets up to five weeks of age are most susceptible, with case fatality rates often reaching 100% in piglets less than two weeks of age.[10,27] Fatality rates progressively decrease as the age of the infected animals increase, with usually only mild or transient signs in uncomplicated infections in nonpregnant adult swine.[10] The most common symptoms observed in adult swine are respiratory signs usually limited to rhinitis with sneezing.[4] PRV can usually be isolated in nasopharyngeal secretions from these swine for up to 17 days after infection.[35] A common sequela of respiratory involvement in pregnant, non-immune sows is abortion, mummified and macerated fetuses or stillborn pigs.[5] Abortion usually occurs 10 to 20 days after onset of the

respiratory signs.[18,33] Although the nasal mucosa is believed to be the primary point of entry of PRV into the pig,[5,19,27,32] venereal transmission from latently infected sows and boars has been suspected.[1,43] Strains of PRV of uniquely high virulence[5,23,25] as well as field strains of low virulence have been reported.[9,14]

Primary viral multiplication at the initial site of infection is most important to the establishment of the disease in the host.[16] In swine, this site is commonly the cells of the nasopharyngeal mucosa, including the palatine tonsils,[27] with the virus later gaining entrance into the central nervous system via cranial nerves innervating the affected region.[36] Other pathways for dissemination of PRV include the lymphatic system,[42,54,55] and the phagocytes of the vascular system.[27] The clearance of virus from the nasal passages could be a result of: 1) mechanical processes found in ciliated epithelial and goblet cells of the nasal passages;[29] 2) a form of cellular immunity commonly detectable in most herpesvirus infections;[44] or 3) the presence of some non-specific protein(s) found in the nasal secretions of swine. This study found evidence to support the last hypothesis, although the other two could not be ruled out.

Virus isolation and identification from any tissues are considered to confirm PRV infection, especially in the presence of clinical manifestations compatable with pseudorabies.[5,8] PRV may be isolated in a variety of cell culture systems by direct inoculation of triturated tissue suspensions[5] or overlay fluids from inoculated tissue fragments,

or co-culture techniques.[8] Viral identification is commonly by serum neutralization (SN) test in cell cultures.[5] Pseudorabies virus is most frequently recovered from turbinates, tonsils, lungs, lymph nodes and spleen of actively infected pigs, and from tonsils, trigeminal nerve ganglia, olfactory bulbs and optic nerves of latently infected animals.[5,8,45] Fluorescent antibody assays for PRV antigens in tissues have also been used diagnostically.[48,51,57] Final confirmation of field pseudorabies infections is usually based on serological studies, such as SN tests on convalescent sera. With presently used tests, convalescent and vaccine induced antibodies cannot be differentiated. Even though neutralizing antibodies are present in sera of nearly all swine with a history of pseudorabies, one study[46] failed to demonstrate the presence of detectable antibodies (specifically IgA) in nasopharyngeal secretions of pseudorabies sero-positive swine.

Early studies on the effects of environmental fomites on pseudorabies virus[50,56] used rabbits as a means of detecting surviving virus. However, later studies have demonstrated that cell cultures may be superior to rabbits for the detection of field strains of PRV. Pseudorabies virus has a wide cell culture susceptibility range[5] and cell culture techniques are more sensitive and reproducible for propagation and detection of infectious virus than are laboratory animal inoculations.[34,36] Based on previous work done at Iowa State University,[13,40] Madin Darby Bovine Kidney cell monolayers were the cells of choice for this study. The use of cell cultures rather than rabbit inoculation

allowed better control of the experiments by reducing the number of unknown variables associated with the use of laboratory animals.

In a recent study at Iowa State University,[12] a clinically recovered sow (PRV infected) was found to be shedding the virus in nasal secretions soon after farrowing, 19 months after its last known infection. Should such an animal have actively shed PRV in an otherwise clean herd, the possibility that an outbreak of pseudorabies would have resulted, would have been dependent upon: 1) the amount of virus excreted into the environment; 2) the ability of the virus to survive in this environment; and 3) contact of susceptible animals to viable virus. The period of viral survival outside the host is highly dependent upon the nature of the fomites upon or into which the virus has been shed.

The purpose of the research reported here was to examine the ability of PRV to remain at infectious levels in environments commonly encountered by pigs, with particular attention being given to those factors which would increase or decrease survival time on or in fomites found in swine raising facilities. Since previous studies[39] have indicated that 75% of outbreaks of pseudorabies in the State of Iowa, studied between 1976-1978, had not resulted from exposure to newly introduced stock. The entrance of contaminated materials onto farms and the ability of PRV to survive on or in environmental fomites and waste handling systems needs to be examined if any meaningful epidemiological investigations of such outbreaks are to be conducted.

In Section I of this thesis, seventeen solid and liquid fomites commonly found in the environment of a hog confinement unit are examined for their effects on the survival of PRV. Tests on these fomites were conducted in such a manner as to closely approximate "ideal conditions" in the environment to enhance viral persistence. Since virus is primarily shed via nasal and oral secretions, these diluents were obtained, using phosphate buffered saline flushes of the nasal cavity for nasal secretions, and injecting pilocarpine hydrochloride to stimulate salivation, especially from parotid salivary glands.[21,49,52] Virus was suspended in these diluents as well as in saline G, a control diluent, and allowed to make contact with the different fomites at a fixed temperature of 25°C (77°F). This temperature was chosen because of its use in previous studies in this laboratory; it is a common temperature in hog confinement units. Virus titers were recorded daily for virus suspended in these diluents, until virus could no longer be detected. The virus and fomites were kept wet during the contact time because drying has been shown to immediately inactivate PRV.[13]

In Section II of this thesis, the survival of PRV in animal waste products and in waste handling systems frequently used in swine operations in the State of Iowa is examined. PRV was suspended in mixtures of these effluents, and virus titers were recorded daily in order to determine inactivation rates and maximum survival times. Results of the study are displayed in tables included within each section and also in graphs found in the Appendix at the end of this thesis.

Regression lines were drawn freehand and slopes were approximated using the available points; however, due to the lack of replications and the small number of measured points, statistical analyses were not performed on the regression parameters. Since the purpose of this study was to determine maximum survival times for PRV in the environment, the data obtained were sufficient to estimate, under ideal conditions at 25°C, the longest periods of time in which PRV would remain at infectious levels for young pigs in the environments tested.

SECTION I: THE EFFECTS OF FEED, WATER AND OTHER ENVIRONMENTAL FOMITES ON THE SURVIVAL OF PSEUDORABIES VIRUS

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ABSTRACT

The survival of pseudorabies virus (PRV) outside the living host was found to be dependent on the diluents and fomite combinations into which the virus was suspended. The maximal survival times expressed in days for PRV at 25°C under moist conditions, when suspended in different diluents in contact with liquid and solid fomites, alone and in combination, were found to be as follows: Swine nasal washings, < 2 days; swine saliva, < 4 days; steel, < 4 days; concrete, < 4 days; polypropylene plastic, < 3 days; vinyl rubber, < 2 days; denim cloth, < 1 day; loam soil, < 6 days; green grass, < 2 days; whole corn, < 4 days; pelleted feed, < 1 day; meat and bone meal, < 2 days; alfalfa, < 1 day; straw, < 4 days; wood, < 2 days; non-chlorinated water, < 7 days; and chlorinated water, < 1 day.

INTRODUCTION

Pseudorabies (PR) (Aujeszky's Disease) has developed into a serious disease of livestock, mostly in swine in Midwestern States since 1969.[38] The areas in which the disease is recognized continue to expand radially from infected foci. Much remains unelucidated in the epidemiology of the infection. This study investigates the ability of the pseudorabies virus (PRV) to remain infectious in the presence of fomites commonly found in the environment of swine raising operations. Previous studies[31,50,56] have indicated that survival of PRV on environmental fomites ranged from 10 to 49 days. However, other studies[13,28] have indicated that survival times more closely approach a week or less. The purpose of this study has been to examine, in a laboratory under controlled conditions, the response of PRV when suspended in different diluents to liquid and solid fomites, alone and in combination.

MATERIALS AND METHODS

Cell Cultures and Cell Culture Media

Confluent 48 hour old Madin Darby Bovine Kidney (MDBK) cell monolayers grown by standard methods in single-use cell culture flasks* or in single-use plates** were used for routine passage and virus titration respectively. Media used were minimum essential medium (MEM)*** with 10% fetal bovine serum (FBS) for cell growth, 2% FBS for cell maintenance, and 0.8% gum tragacanth with a 2% FBS for overlay medium.[13] Antibiotics were added to all media at the rate of 100 units/ml of penicillin G; 100 micrograms/ml of streptomycin sulfate; 3 micrograms/ml of amphotericin B; and 5 mg/ml of gentamycin sulfate.[40]

Virus and Virus Propagation

The strain of pseudorabies virus used throughout this study was an eighth laboratory MDBK cell culture passage of an Iowa field isolate called S62/26 or the "Wilson" strain.[13] Subjectively, the virus was considered to be moderately virulent. Twenty ml aliquots of the virus at $10^{7.3}$ plaque forming units (PFU) per ml were stored at -90°C until used.

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*Corning Glass Works, Corning, New York.

**Lux Plates, Lux Scientific Corporation, Newbury Park, California.

***Grand Island Biological Company, Grand Island, New York.

Viral Assay

Viral titers were assayed utilizing a plaque counting technique. Serial dilutions of virus were made in cold saline G.[13] One ml of each virus dilution was inoculated onto 48 hour old MDBK cell monolayers in Lux cell culture plates. Inocula were removed after 90 minutes and replaced with 2 ml of overlay medium. Virus-infected cell sheets were incubated for 48 hours at 37°C in a 5% CO_2 atmosphere. Afterwards, the monolayers were fixed with 20% formalin and stained with 5% aqueous crystal violet solution. Viral titers were calculated by counting the plaques, converting to $logs_{10}$, and finally, expressing the titers as the number of plaque forming units (PFU) per milliliter of inoculum.

Animals

One hundred weanling pigs from pseudorabies-free sows yielded the saliva and nasal washings needed as diluents. Serum samples from these pigs were negative for pseudorabies virus neutralizing antibodies.

Diluents

Pseudorabies virus is commonly found in the nasal-oral secretions of PRV-infected swine actively excreting the virus.[53] Therefore, saliva and nasal washings were used as the primary diluents in which virus was suspended for tests in contact with environmental fomites. Saline G, which has little inhibitory effect on pseudorabies virus at 25°C,[13] was

used as a control diluent for comparison of findings with saliva or nasal washings.

Saliva was obtained by injecting weanling pigs with 1% pilocarpine hydrochloride solution, causing stimulation of salivation. Saliva samples were poured into a single sterile 1 liter container resulting in a "pooled" mixture of the secretions.

Nasal washings were obtained by flushing each nostril of weanling pigs with 5 cc of a phosphate buffered saline (PBS) solution, pH 7.0. As before, the samples were poured into a single, sterile, 1 liter container resulting in a "pooled" mixture of the washings. The containers of saliva and nasal washings were placed in a refrigerator overnight, allowing foreign particulate matter to settle to the bottom, resulting in a clearer product. The desired supernate was removed, antibiotics were added at the same level as for cell culture media, and the final products were stored in 100 ml aliquots at -4°C until used. Both saliva and nasal washings were free of pseudorabies antibodies as evidenced by negative indirect fluorescent antibody tests.

Fomites

A fomite may be defined as any inanimate object or non-living substance that may be contaminated with infectious organisms, thus serving as a vehicle for disease transmission. The fomites chosen for this study were those frequently occuring in swine-raising environments. The fomites used are listed in Table 1.

Table 1. Fomites and source

Fomite	Source
Steel	Stainless Steel Bucket
Concrete	Concrete Flooring
Polypropylene Plastic	Plastic Milk Jug
Vinyl Rubber	Rubber Boots
Denim Cloth	Jeans
Loam Soil	Iowa
Green Grass	Iowa
Whole Corn	Iowa
Pelleted Feed	Both "Starter" and "finishing" Feed Sampled
Meat and Bone Meal	Rendering Plant
Alfalfa	Iowa
Straw	Iowa
Wood (Pine)	Swine Bedding
Chlorinated Water	Iowa City: Ames
Well Water	Iowa Counties: Adair, Cass, Story

Procedures

Pseudorabies virus suspensions, fomites and diluents (saline G, nasal washings or saliva) were mixed in the ratio of 1:1:8, v/w/v, respectively. The mixtures were then stored in a 25°C incubator and sampled daily for PRV. The pH was measured at the beginning and also at the end of each test. Tests were performed with fomites under moist conditions, since previous studies[13] indicated drying to be very deleterious to survival of PRV. A constant temperature of 25°C was used throughout the study, not only to reduce the number of variables, but also because it is a common temperature in closed confinement units for swine. Titers were recorded at time 0, 1 day, 2 days, 3 days, 4 days, 7 days, and every 3rd day thereafter, when necessary. Initial viral titers up to $10^{7.3}$ were recorded in this study. These titers are similar to maximal viral levels in tissues of infected animals.[47] Final viral titers of 10^{1} were considered to be the minimum infectious level for 2 week-old pigs.[3,7]

RESULTS

The effects of diluent alone on the persistence of PRV are listed in Table 2.

The effects of diluent/fomite mixtures on the persistence of PRV are listed in Tables 3, 4 and 5.

The effects of non-chlorinated and chlorinated water on the persistence of PRV are listed in Table 6.

Table 2. The effects of diluents on the titers of pseudorabies virus

Diluent	Initial Titer (Log)	Initial pH	Final Titer (Log)	Final pH	No. of Days to Final Titer	Drop in Titer (Logs/Day)
Saliva	7.3	8.3	< 1	8.4	< 4	-1.7
Nasal Washing	7.3	7.2	< 1	7.4	< 2	-3.5
Saline G	7.3	7.0	6.5	7.0	10	< -0.1
PBS	7.3	7.0	6.6	7.0	10	< -0.1

Table 3. The effects of saline G plus fomites on the titers of pseudorabies virus

Fomite	Initial Titer (Log)	Initial pH	Final Titer (Log)	Final pH	No. of Days to Final Titer	Drop in Titer (Logs/Day)
Steel	7.3	7.0	4.6	7.0	7	-0.4
Concrete	6.6	10.5	< 1	11.5	< 1	-6.6
Polypropylene Plastic	7.3	7.0	< 1	6.1	< 7	-0.9
Vinyl Rubber	7.2	6.8	< 1	7.5	< 7	-1.0
Denim Cloth	7.2	7.1	< 1	6.7	< 1	-7.2
Loam Soil	7.3	7.0	< 1	6.2	< 4	-1.9
Green Grass	7.2	6.7	< 1	5.7	< 1	-7.2
Whole Corn	7.3	6.9	5.9	6.1	7	-0.2
Pelleted Feed	7.3	6.1	< 1	5.5	< 3	-2.4
Meat and Bone Meal	7.2	6.5	< 1	6.1	< 5	-1.5
Alfalfa	6.1	6.2	< 1	5.7	< 1	-6.1
Straw	7.3	7.0	< 1	6.5	< 3	-2.4
Wood	6.0	5.0	< 1	5.0	< 1	-6.0

Table 4. The effects of nasal washing plus fomites on the titers of pseudorabies virus

Fomite	Initial Titer (Log)	Initial pH	Final Titer (Log)	Final pH	No. of Days to Final Titer	Drop in Titer (Logs/Day)
Steel	7.3	7.4	< 1	7.1	< 2	-3.6
Concrete	7.3	10.0	< 1	11.4	< 1	-7.3
Polypropylene Plastic	7.3	7.2	< 1	7.2	< 3	-2.6
Vinyl Rubber	7.2	7.3	< 1	7.5	< 2	-3.6
Denim Cloth	7.2	7.4	< 1	7.3	< 1	-7.2
Loam Soil	7.3	7.0	< 1	6.5	< 2	-3.6
Green Grass	7.2	7.0	< 1	6.2	< 1	-7.2
Whole Corn	7.3	7.4	< 1	6.1	< 2	-3.6
Pelleted Feed	7.3	6.7	< 1	5.8	< 1	-7.3
Meat and Bone Meal	7.2	6.6	< 1	6.4	< 2	-3.6
Alfalfa	6.6	6.5	< 1	5.7	< 1	-6.6
Straw	7.3	7.2	< 1	6.8	< 4	-1.9
Wood	6.7	6.3	< 1	6.3	< 1	-6.7

Table 5. The effects of saliva plus fomites on the titers of pseudorabies virus

Fomite	Initial Titer (Log)	Initial pH	Final Titer (Log)	Final pH	No. of Days to Final Titer	Drop in Titer (Logs/Day)
Steel	7.3	8.4	< 1	8.0	< 4	-1.8
Concrete	7.3	9.5	< 1	9.2	< 4	-1.8
Polypropylene Plastic	7.3	8.2	< 1	8.0	< 3	-2.6
Vinyl Rubber	7.2	8.2	< 1	8.3	< 2	-3.6
Denim Cloth	7.2	8.2	< 1	7.7	< 1	-7.2
Loam Soil	7.3	8.2	< 1	7.2	< 7	-1.0
Green Grass	7.2	7.8	< 1	7.0	< 2	-3.6
Whole Corn	7.3	8.2	< 1	7.4	< 4	-1.7
Pelleted Feed	7.3	7.5	< 1	6.8	< 1	-7.3
Meat and Bone Meal	7.2	7.1	< 1	7.2	< 2	-3.6
Alfalfa	7.2	7.2	< 1	7.1	< 1	-7.2
Straw	7.3	8.2	< 1	7.4	< 4	-1.7
Wood	6.6	7.1	< 1	7.8	< 2	-3.3

Table 6. The effects of water on the titers of pseudorabies virus

Fomite (Water)	Initial Titer (Log)	Initial pH	Final Titer (Log)	Final pH	No. of Days to Final Titer	Drop in Titer (Logs/Day)
Non-Chlorinated (Well)						
Adair	4.3	6.7	< 1	6.7	< 2	-2.2
Cass	4.3	6.8	< 1	6.8	< 3	-1.4
Story	4.3	7.1	< 1	7.4	< 7	-0.6
Chlorinated (City) Ames	4.3	7.6	< 1	7.7	< 1	-4.3

DISCUSSION

Effects of Diluents

Saline G and PBS

The rates of inactivation of pseudorabies virus in either saliva or nasal washings were very high when compared to the rates of inactivation in either saline G (< 0.1 log/day) which was used as a standard diluent in a previous study,[13] or in PBS (< 0.1 log/day). The inactivation rates of PRV suspended in each of the test diluents in the presence of the test fomites were compared, with the rates in saline G and PBS as control diluents. When a fomite mixed with saline G or PBS yielded a greater inactivation rate than with saline G or PBS alone, it was considered that the additional inactivation was due to the fomite. Inactivation rates recorded for PRV suspended in saliva or in nasal washings in the presence of the same fomites were compared to those for the virus in saline G or PBS, to assess the effect of each fomite as protecting the virus from the inactivating effects of saliva or nasal washings, as having no effect on the inactivation rates or as increasing the inactivation rates in the test diluents.

Nasal washings

Pseudorabies virus was inactivated in less than 2 days suspended in nasal washings, consisting of nasal secretions in PBS. The average inactivation rate was 3.5 logs/day. Since PBS alone had a low inactivation

rate on the virus, it was concluded that the presence of antiviral factors in nasal exudates was responsible for the rapid inactivation of PRV. This conclusion complimented hypotheses put forth in a previous study[29] indicating that clearance of infectious bovine rhinotracheitis (IBR) virus, a member of the herpesvirus family, from the nasal passages of non-immune calves could be attributed to normal nasal secretions and cilliary action of the mucosa, antiviral factors in the secretions, or a combination of both.

Saliva

Pseudorabies virus was inactivated in less than 4 days when suspended in swine saliva. The average inactivation rate was 1.7 logs/day. The pH of the saliva was 8.3, which according to a previous study,[13] was not in the range to account for such rapid inactivation of PRV at 25°C. It was considered that saliva, like nasal washings, contained antiviral factors which affected the envelope of the virus, preventing attachment to susceptible cells. The presence of mucous secretions in saliva may have contributed to its ability to act as a buffer,[21] explaining the protective effect of this diluent, which, in the presence of certain fomites, was greater than that afforded by saline G.

Effects of Diluents with Fomites

Steel

The addition of steel to the diluents did not result in an appreciable change in the inactivation rate of the virus as compared to the inactivation rate with the diluents alone. Materials made of steel, contaminated with pseudorabies virus could remain a source of infection for pigs less than 2 weeks-old for 2-4 days at 25°C.

Concrete

The addition of concrete to saline G and nasal washings increased the inactivation rate of the virus to 6.6 and 7.3 logs/day, from < 0.1 and 3.6 logs/day respectively. As in a previous study,[13] virus titer rapidly dropped in alkaline solutions of pH 9.0 or greater. Concrete changed the pH of the diluents to 9.5-11.5. In the presence of nasal washings and concrete, PRV could be inactivated in less than 24 hours at 25°C.

Saliva, however, was able to exert a protective effect, holding the inactivation rate of the virus in contact with concrete to 1.8 logs/day, essentially the same as the virus in saliva alone, which was 1.7 logs/day. Virus in saliva on concrete under these conditions could remain infectious for pigs less than 2 weeks-old for 3-4 days, whereas virus in nasal washings would decay below infectious levels in less than 1 day.

Polypropylene plastic

Widely used in the manufacture of plastic-coated floor mesh and other items, polypropylene in saliva and nasal washings changed the inactivation rate of PRV to 2.6 logs/day, from 1.7 and 3.6 logs/day, respectively. The addition of the plastic to saline G changed the rate from < 0.1 to 0.9 logs/day, indicating that this fomite contains antiviral properties. It was concluded that PRV, in either saliva or nasal washings, could remain viable at levels infectious to 2 week-old pigs for up to 3 days in the presence of this fomite at 25°C.

Vinyl rubber

Widely used in the manufacture of rubber boots, vinyl rubber in saliva increased the inactivation rate of PRV to 3.6 logs/day, from 1.7 logs/day. In the presence of saline G the rate was changed from < 0.1 to 1.0 logs/day and in nasal washings the inactivation rate remained the same. Although showing some antiviral properties, it was concluded that titers infectious to 2 week-old pigs could persist for up to 2 days in the presence of this fomite at 25°C.

Denim cloth

The addition of denim cloth to the diluents caused rapid inactivation of the virus. The average inactivation rate of PRV in all test diluents due to the addition of this fomite was 7.2 logs/day. Since the cloth sample was taken from a frequently worn pair of jeans, it was difficult

to assess whether residues from washing or other antiviral factors added to the cotton were present on the sample. A previous study[28] did not indicate cotton fibers, in themselves, to be very viricidal. The ability of pseudorabies virus to survive on denim cloth would be dependent on whether or not the antiviral agents on this material can come in contact with the virus. Filth, mucous or some other protective material could enable the virus to remain viable long enough to infect susceptible swine. From this study it was concluded that the rapid inactivation of PRV in contact with denim cloth was independent of the diluent in which the virus was suspended, and viral titers dropped below infectious levels to even 2 week-old pigs in less than 24 hours.

Loam soil

A mixture of loam soil and saliva provided a medium which seemed to slightly enhance viral persistence. The average rate of viral inactivation was 3.6 logs/day in nasal washings in contact with soil, unchanged from nasal washings alone, and 1.0 logs/day in saliva in contact with soil compared to 1.7 logs/day in saliva alone. Loam soil thus acted somewhat as a protection to virus suspended in saliva, extending the time of infective dose level for 2 week-old pigs from 3-4 days to 5-6 days.

Green grass

A mixture of green grass and diluents provided a medium which seemed to increase the inactivation rate of PRV. The addition of green

grass to saline G and nasal washings increased the inactivation rate of the virus to 7.2 logs/day, from < 0.1 and 3.6 logs/day respectively. Saliva and green grass increased the inactivation rate to 3.6 logs/day as compared to an inactivation rate of 1.7 logs/day in saliva alone.

Virus in saliva on green grass could remain infectious for pigs less than 2 weeks-old for 1-2 days at 25°C. However, virus in nasal washings on green grass would fall below infectious levels in less than 24 hours.

Whole corn

The addition of whole corn to the diluents did not appreciably affect the rate of inactivation due to antiviral factors found in the diluents alone. The ability of the virus to persist on moist corn at levels infectious to 2 week-old pigs ranged from 2-4 days.

Pelleted feed

In this study, both "starter" and "finisher" feed were evaluated. When mixed with nasal washings or saliva, each caused the same rapid inactivation rate of the virus, the rate of inactivation being 7.3 logs/day (compared to rates of 3.6 and 1.7 logs/day, respectively, for the diluents alone). In saline G, the rate was 2.4 logs/day. Feed contaminated with PRV in nasal or oral secretions would not be expected to remain a source of infection for 2 week-old or older pigs after several hours at 25°C.

Meat and bone meal

The addition of meat and bone meal to saline G and saliva slightly increased the inactivation rate from 0.1 to 1.5 logs/day and from 1.7 to 3.6 logs/day respectively. In nasal washings, the inactivation rate (3.6 logs/day) remained the same. The persistence of PRV in the presence of this fomite, at levels infectious to 2 week-old pigs up to 48 hours at 25°C, however, still makes this product a possible vehicle of infection.

Alfalfa

The addition of alfalfa to saline G, nasal washings and saliva increased the inactivation rate of the virus to 7.2 logs/day, from < 0.1, 3.6 and 1.7 logs/day respectively. Like green grass, the rapid inactivation of PRV in contact with alfalfa indicated inactivating activity independent of the diluents used. Virus in diluent on alfalfa would fall below the infectious level for 2 week-old pigs in less than 24 hours at 25°C.

Straw

Commonly used as bedding material, straw did not display the same antiviral properties exhibited by the grasses and legumes high in chlorophyl. The rates of inactivation in the three test diluents were 1.7-2.4 logs/day, indicating a protective effect on virus in nasal washings, no effect on virus in saliva but increasing inactivation of the virus in saline G. It was concluded that PRV in either saliva or

nasal washings could remain viable at levels infectious to 2 week-old pigs for up to four days in the presence of this fomite at 25°C.

Wood

Widely used as flooring, walls, bedding or other contact surfaces, new wood displayed marked antiviral properties when added to the diluents. The rate of inactivation was 7.2 logs/day in nasal washings and saline G and 3.3 logs/day in saliva. In nasal washings on wood, the virus titer decreased below the infectious level for 2 week-old pigs within 1 day and in saliva within 2 days, indicating a strong inactivating factor(s) found in the test wood. Since freshly milled wood was used in this experiment, resins present in the wood may have been the "inactivating factor(s)." Again, further studies would be needed to prove this hypothesis. It is recommended that in PRV infected herds, if bedding material is utilized, it would appear that fresh wood shavings or sawdust would be preferred over other types of bedding until outbreaks are over.

Effects of Water

Well water and chlorinated city water

At 25°C, pseudorabies virus was able to remain viable in non-chlorinated well water from 2-7 days, the rates of inactivation being from .6 to 2.2 logs/day. The chemical composition of the water samples may have contributed to the variance in viral survival times. In any

case, well water in small containers as drinking cups or pans could be considered a source of infection if contaminated with large amounts of virus. Wells and larger water reservoirs would lead to immediate dilution below infectious doses, even if directly contaminated with PRV and thus would not act as effective fomites.

Chlorinated water from the city of Ames, Iowa, used as a comparative sample, inactivated the virus below a detectable level in less than one day. This confirmed another study[30] which recommended the use of calcium hypochlorite for rapid destruction of Aujeszky's disease virus in contaminated waters.

SECTION II: THE EFFECTS OF FECES, URINE, AND WASTE HANDLING SYSTEMS ON THE SURVIVAL OF PSEUDORABIES VIRUS

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ABSTRACT

The ability of pseudorabies virus (PRV) to persist in fresh animal waste products or in animal wastes in waste handling systems was dependent upon the presence of feces and related products in contact with the suspended virus. The maximum survival time for PRV at 25°C, under moist conditions, when suspended in samples of fresh swine feces, or effluent from swine pits or lagoons, was less than 2 days, 1 day, or 2 days, respectively. Sow urine, uncontaminated with fecal products, maintained titers infectious for 2 week-old pigs for up to 14 days, at 25°C.

INTRODUCTION

An accurate knowledge of the effects of environmental factors upon the ability of pseudorabies virus (PRV) to survive outside the living host is essential for developing effective control of transmission of the disease in swine. Important among such environmental fomites are urine and feces, and waste handling systems in swine-raising operations. Virus recovery from feces and urine of infected swine has been reported,[6,11,26,37] and studies[2,41,56] have further indicated that PRV was able to remain viable in such urine or feces for several days at summer temperatures.

The purpose of this study was to examine, under laboratory controlled conditions, the response of pseudorabies virus when suspended in animal waste products and in effluent from waste handling systems, both held at a constant temperature of 25°C.

MATERIALS AND METHODS

Cell Cultures and Cell Culture Media

The cell culture system utilized was a Madin Darby bovine kidney (MDBK) cell line grown by standard methods in single-use culture flasks* for viral passage or in single-use plates** for viral titration. The media utilized were minimum essential medium (MEM)*** with 10% fetal bovine serum (FBS) for cell growth, 2% FBS for cell maintenance and 0.8% gum tragacanth with 2% FBS for overlay medium.[13] Antibiotics were added to all media, including saliva and nasal washings, at the rate of 100 units/ml of penicillin G; 100 micrograms/ml of streptomycin; 3 micrograms/ml of amphotericin B; and 5 mg/ml of gentamycin.[40]

Virus and Virus Propagation

The virus strain used throughout the study was an 8th laboratory passage in cell culture of an isolate obtained from swine in a field outbreak in Iowa. Known as S62/26 or the "Wilson" strain, it has been assessed as causing moderately severe disease in pigs.[13]

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*Corning Glass Works, Corning, New York.

**Lux Plates. Lux Scientific Corporation, Newbury Park, California.

***Grand Island Biological Company, Grand Island, New York.

Viral Assay

Viral titers were assayed by using a plaque counting technique. After making serial dilutions of virus in cold saline G, one ml of each dilution was inoculated onto MDBK monolayers.[13] Inocula were removed after 60 minutes and replaced with 2 ml of overlay medium. Following incubation, cell monolayers were fixed with 20% formalin and stained with 5% aqueous crystal violet solution. PRV titers were determined by counting the plaques, converting to $\log_{10}$, and expressing the titers as the number of plaque forming units (PFU) per ml of inoculum.

Fomites

In order to evaluate the ability of pseudorabies virus to survive in animal wastes and in waste handling systems, representative samples for study were taken from PRV-free swine or swine-raising facilities. Freshly voided feces were collected from feeder pigs. Urine was obtained from sows via catheterization. Effluent was taken from a swine pit and from three anaerobic lagoons when the mean effluent temperature was 25°C. Effluent samples were collected from the surface, middle and bottom layers of each lagoon in order to study the effect of water depth on viral inactivation.

Diluents

Swine saliva and nasal washings were used as the primary diluents in which PRV was suspended for tests on viral survival in contact with feces. Saliva and nasal washings were from the same stock used in the study of fomites described in Section I.

Procedures

In studies using feces, PRV, swine feces and diluents were mixed in the ratio of 1:1:8, v/w/v, respectively. In studies using urine, PRV was directly suspended in swine urine at the ratio of 1:9. In studies using effluent, the virus was added to pit or lagoon liquids to give a test concentration of virus of approximately 10^4. All mixtures were tested for survival at 25°C. The pH was measured at the beginning and end of each test. Titers were recorded for each mixture at time 0, 1 day, 2 days, 3 days, 4 days, and every third day thereafter as long as the virus could be detected. Initial viral titers were approximately 10^7 for tests on feces and urine and 10^4 for tests on pit and lagoon effluent. Final viral titers of 10^1 were considered to be the minimum infectious level for 2 week-old pigs.[3,7]

RESULTS

The effects of animal wastes and waste handling systems on the persistence of PRV are listed in Table 7.

Table 7. The effects of feces, urine, pit effluent, and lagoon effluent on pseudorabies virus

Fomite	Initial Titer (Log)	Initial pH	Final Titer (log)	Final pH	No. of Days to Final Titer	Drop in Titer (logs/day)
Feces Suspended in:						
Nasal Washings	7.3	7.7	< 1	6.7	< 2	-3.6
Saliva	7.3	6.6	< 1	6.0	< 2	-3.6
Urine						
Sow 1	7.3	8.5	< 1	8.3	< 5	-1.5
Sow 2	7.3	8.2	< 1	8.2	< 10	-0.7
Sow 3	7.3	8.3	< 1	8.2	< 14	-0.5
Pit effluent	3.6	5.8	< 1	6.0	< 1	-3.6
Lagoons (Iowa)						
Adair County	4.0	7.8	< 1	7.6	< 2	-1.9
Cass County	4.0	7.7	< 1	7.6	< 2	-1.9
Story County	4.0	7.3	< 1	7.4	< 2	-1.9

DISCUSSION

Effects of Fresh Animal Wastes

Feces

The addition of feces to saliva or nasal washings resulted in a uniform inactivation rate of 3.6 logs/day.

Bile salts are one of the elements found in swine feces. Since these salts are capable of emulsifying fats and reacting with lipids,[22] it is possible that an enveloped virus such as PRV could be susceptible to such factors and therefore would not survive well in the alimentary tract, as reported by other authors.[5,15] To investigate this, PRV was mixed with various dilutions of swine bile. It was observed that 10^3 plaque forming units of PRV could be inactivated by a 1:100 dilution of porcine bile in less than 1 hour, or by a 1:1000 dilution in less than 24 hours. Further studies along these lines would be needed to strengthen this hypothesis.

PRV in saliva or nasal washings in contact with feces could remain at infectious levels for 2 week-old pigs for, at most, 2 days at 25°C.

Sow urine

PRV suspended in sow urine was able to persist at levels which could be infectious for 2 week-old pigs from 3 to 14 days. The inactivation rate ranged from 0.5 to 1.5 logs/day, indicating that at 25°C, urine is less inhibitory to PRV than swine saliva (1.8 logs/day) or nasal washings (3.6 logs/day). Although PRV had previously been reported by European workers in kidneys and urine,[5] a more recent study by

American researchers[14] reported the isolation of PRV from the kidney of a 1 month-old pig, experimentally infected. The demonstration of PRV in the kidneys implicates urine as a means whereby the virus can gain access to the external environment, possibly infecting susceptible animals.

Effects of Animal Wastes in Waste Disposal Systems

Swine pit effluent

Swine pits, commonly 4 to 5 feet deep, are constructed under confinement units to collect waste products for later disposal. The addition of PRV to a sample of this effluent resulted in a reduction of viral titer below detectable levels in less than 1 day. Since such effluent is a composite of feces, urine, feed, secretions, and other sources of potential virus inhibiting factors, it was difficult to assess the causative agents or combinations thereof which inactivated the virus. From this study, however, it was determined that pit effluent at 25°C reduced PRV levels below levels which would be infectious for 2 week-old pigs in less than 24 hours.

Swine lagoons

Anaerobic lagoons, usually 60-100 feet long and 8-12 feet deep are frequently used in the State of Iowa to collect and decompose swine wastes. Three lagoons in Adair, Cass and Story counties respectively, were sampled to determine if lagoons could act as reservoirs of

infection for pseudorabies disease. Regardless of the water depth or lagoon sampled, PRV was inactivated at a rate of 1.9 logs/day in these structures. Interestingly, this rate was approximately 1/2 the inactivation rate in the suspension of feces, possibly indicating the effect of diluting feces and thus of their antiviral components with water. If the inflow of PRV into lagoons came from only a few shedding pigs, there would be immediate great dilution of the virus, possibly below infectious levels for even the smaller pigs, but if large numbers of pigs were shedding, the lagoons could become heavily contaminated. Since lagoons are utilized in reflush systems for hog confinement units, the potential for reintroducing the virus back into the unit is there for up to 2 days, at 25°C, after which time the titer would drop below detectable levels.

GENERAL SUMMARY AND DISCUSSION

The experimentation reported in this thesis was an attempt to simulate and examine under controlled laboratory conditions, environmental factors which would influence the ability of pseudorabies virus (PRV) to persist outside the swine host, and thus record which of those factors were the most favorable or inhibitory to virus survival.

It was first demonstrated that nasal washings, saliva and even urine could act as vehicles of transmission for PRV from infected animals to the external environment. These liquid fomites alone were able to support viral titers for days, and in combination with other solid fomites, could either increase or decrease the duration of viral survival. The goal of good swine management should include the manipulation of those factors in the external environment which would inhibit entrance onto the farm or spread within the farm of PRV.

Plastic and metal structures, particularly of stainless steel and polypropylene, provided surfaces upon which PRV can remain infectious for several days at 25°C. Virus-contaminated equipment composed of these materials would be a potential source of infection to susceptible livestock, especially the younger animals.

In contrast to steel or plastic, clean concrete showed antiviral properties, with rapid elevation of pH to 11 or higher. However, if the virus does not directly contact the surface of concrete because of the presence of intervening filth, buffering or acidic liquids, or other

suspending materials, then the virus could, as before, persist for days. In order to take advantage of the antiviral properties of concrete, a thorough periodic scrubbing of floors, walls, pens and other structures made of this material would keep the surface of concrete exposed, better facilitating viral contact with concrete.

In most swine operations, personnel frequently use "foot baths," scrubbing and disinfecting their boots before entering or leaving housing units. Considered by most to be a proper method of cleaning and disinfection (C&D), closer examination of this technique reveals most people clean only about 10 to 90% of their boots, ignoring the tops of the boots, especially the areas where the boots and trousers join. Two inches above and below this boot-trouser junction is an ideal site for deposition of virus, since this area is frequently "sniffed and nibbled" whenever one walks among pigs. PRV suspended in nasal secretions or saliva can survive for days on rubber or cloth; thus boots contaminated with PRV in 1 unit or on 1 farm could transport the virus to another unit or farm if proper steps are not taken to remove these contaminants. One method to prevent transmission of virus between units or farms would be to simply change clothes and boots between visits. However, this would require quite an inventory of wearing apparel. Another preventive measure would be the use of a "fogger" filled with a quaternary ammonium solution to fog clothes and boots after cleaning off manure, etc. with detergent. This technique is frequently used in poultry operations with great success, and requires little extra effort on the

part of animal caretakers. Clothes or boots contaminated with PRV can be easily disinfected with a fogger without necessitating the constant changing of clothes.

Swine feed or conveyances of feed have been suspected in the transference of PRV from one herd to another. Based on the results obtained in this study, only whole corn demonstrated the potential to act as an effective source of infection of pseudorabies. Pelleted feed, and especially feed which contained alfalfa, a potent PRV inhibitor, rapidly inactivated the virus. Virus contaminated feed would not support infectious levels of PRV for more than a few hours at 25°C, with the exception of whole corn, which under conditions of this study, could maintain titers of PRV infections to young pigs for up to 4 days. A more probable transport for the virus would be in the vehicles that haul the feed, especially, if previous to the feed, the truck had been used to haul pigs actively shedding PRV. It is possible that feed would then become exposed to PRV but again, it would have to be fed to hogs within hours to successfully transfer the virus to susceptible animals.

Bedding materials, such as straw, wood shavings, or saw dust have also been considered as fomites which could spread PRV. Placed in areas where virus was being shed, bedding material would indeed become contaminated with PRV; however, only straw demonstrated the potential to act as an important vehicle of transmission for PRV. Wood shavings and sawdust so rapidly inactivated the virus that their use could be

recommended in housing units or conveyances where transport of PRV might be a problem. Straw enables the virus to persist for days, making it possible that it could effectively transfer the virus to susceptible animals that nibble or sniff this material. Soiled straw should never be reused or allowed to be contacted by replacement or new animals. Prompt removal of this material, followed by a thorough C&D of the premises, is recommended wherever straw is used.

In opposition to closed-confinement units, open lots or pastures are also utilized in swine rasing operations. Pseudorabies, although able to persist in the soil for several days under certain conditions, would be rapidly inactivated in the presence of sunlight and green grass, at 25°C. Soil that is barren of vegetation, shaded from direct sunlight, and frequently kept moist, could present a greater threat of PRV exposure than soil in range areas. As in other management recommendations, standing water of "mud wallows" should be drained or removed, especially if shaded from the sun. Hog lots should be kept as dry as possible, allowing moist areas to be exposed to the direct rays of the sun.

One of the most prevalent fomites in the environment of a hog raising operation is manure, and with each farm, there is a system constructed to deal with this problem. In this study, it was concluded that PRV could be able to survive in manure and in lagoons for up to 2 days, but would be inactivated within a matter of hours in swine pits. Urine alone could allow the virus to persist for days, but once contaminated with feces would reduce the survival time to 2 days. From

the results obtained in this study, it was concluded that at 25°C, swine pits would be a very unlikely source of PRV to susceptible swine. Even if feces were shed containing PRV, the effluent in the pit would render the virus non-infectious and thus should not be considered a source of infection during an outbreak. Lagoons, on the other hand, could support the virus long enough to be a problem, especially if the effluent were recycled to wash confinement units in a reflush system. Virus contaminated lagoon water, flushed through a closed confinement unit could aerosolize the virus, exposing susceptible swine. However, should the lagoon water be allowed to stand for at least 2 days before recycling, the levels of virus in the effluent would be reduced to a point below infective doses. Pumping hoses and vehicles used to pump out lagoons could become contaminated with PRV, as could, of course, the personnel operating the equipment. Farm operators should require these individuals to C&D themselves and their external equipment before entering a hog raising operation, especially in an area endemic with pseudorabies.

To summarize, since PRV can survive in a manure contaminated environment for up to 2 days, areas exposed to virus contaminated feces should be thoroughly cleaned, scrubbed and disinfected before the addition of susceptible pigs.

Water is used in many ways in a swine-raising operation, from drinking to cleaning, and swine have extensive contact both with clean and contaminated water. From this study, it was determined that the use

of chlorinated as opposed to the use of non-chlorinated water should be recommended. The chlorine level in the Ames municipal water used in this test, .93 ppm, was sufficient to inactivate the virus in less than 1 day. Non-chlorinated water, however, could support infectious levels of PRV for days. This could have serious implications in a closed confinement unit where animals share common watering and waste disposal facilities. In PRV endemic areas, operators should chlorinate their well water as a measure to reduce the chances for spreading the virus.

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APPENDIX

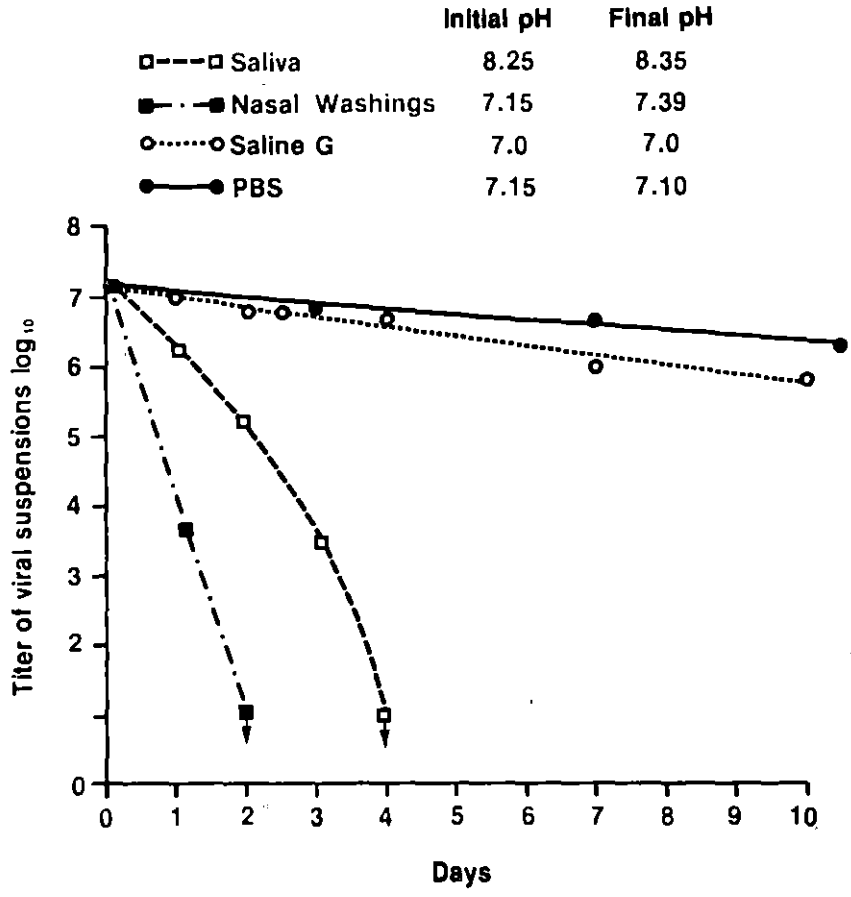

Figure 1. The effects of diluents on the titers of pseudorabies virus

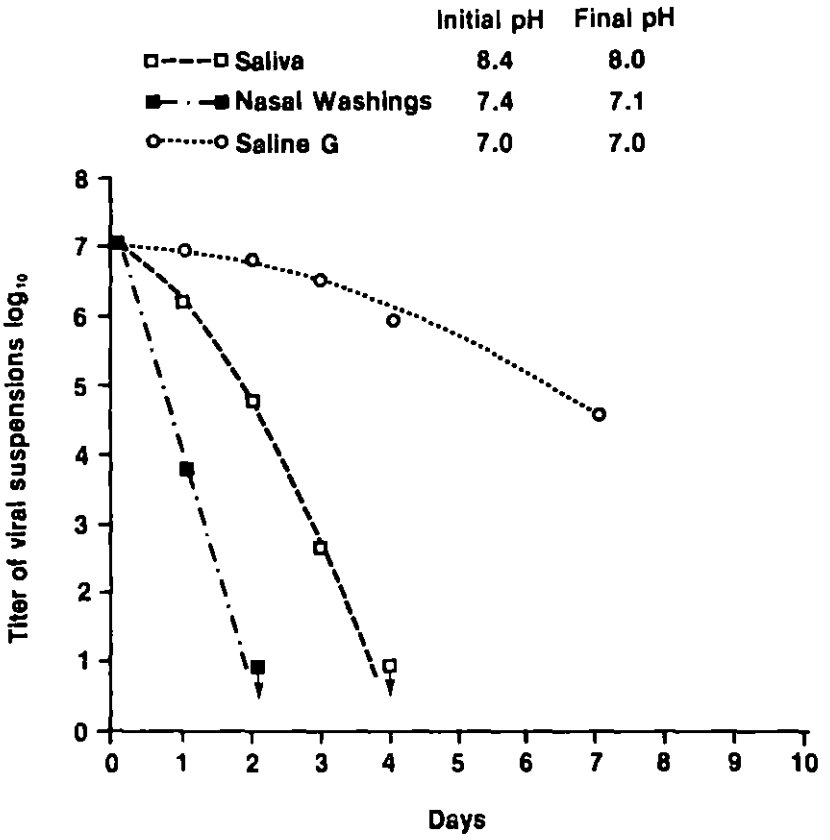

Figure 2. The effects of steel/diluents on the titers pseudorabies virus

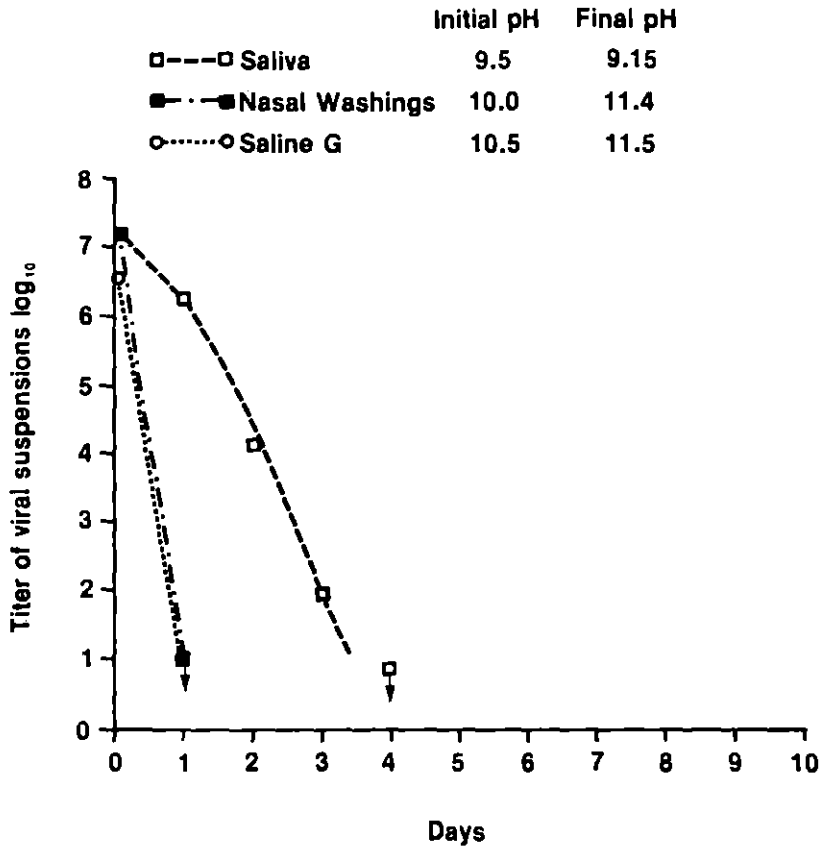

Figure 3. The effects of concrete/diluents on the titers of pseudorabies virus.

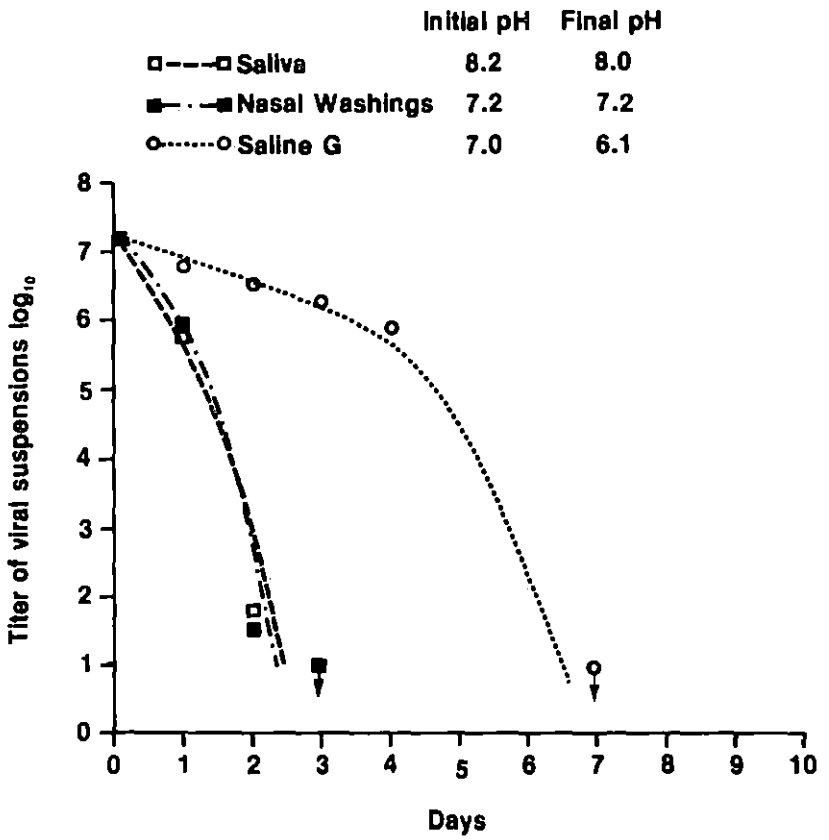

Figure 4. The effects of polypropylene/diluents on the titers of pseudorabies virus

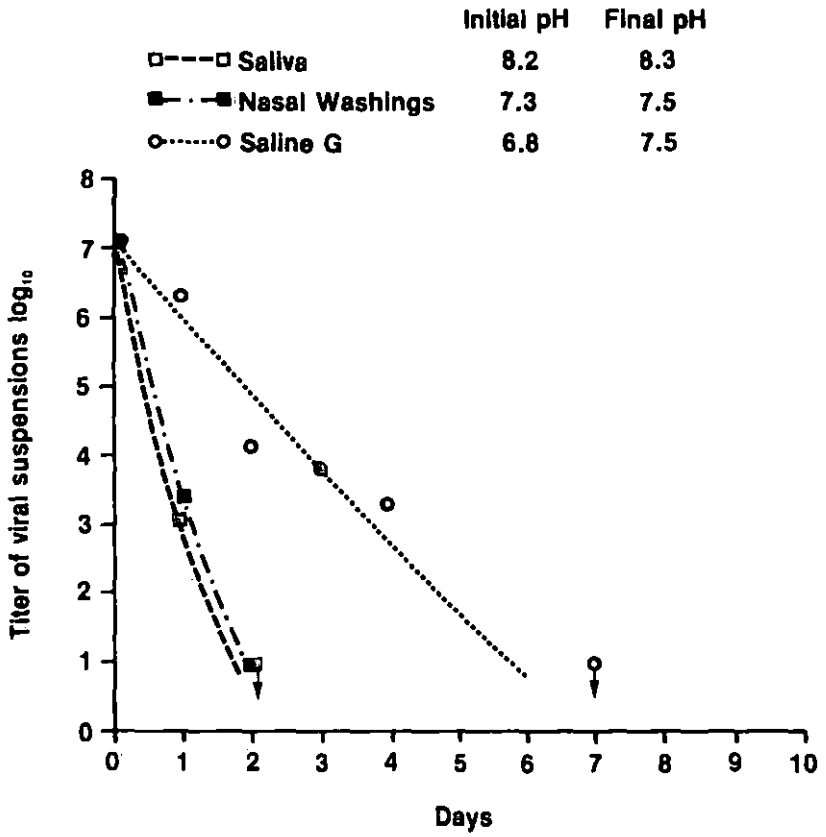

Figure 5. The effects of vinyl rubber/diluents on the titers of pseudorabies virus

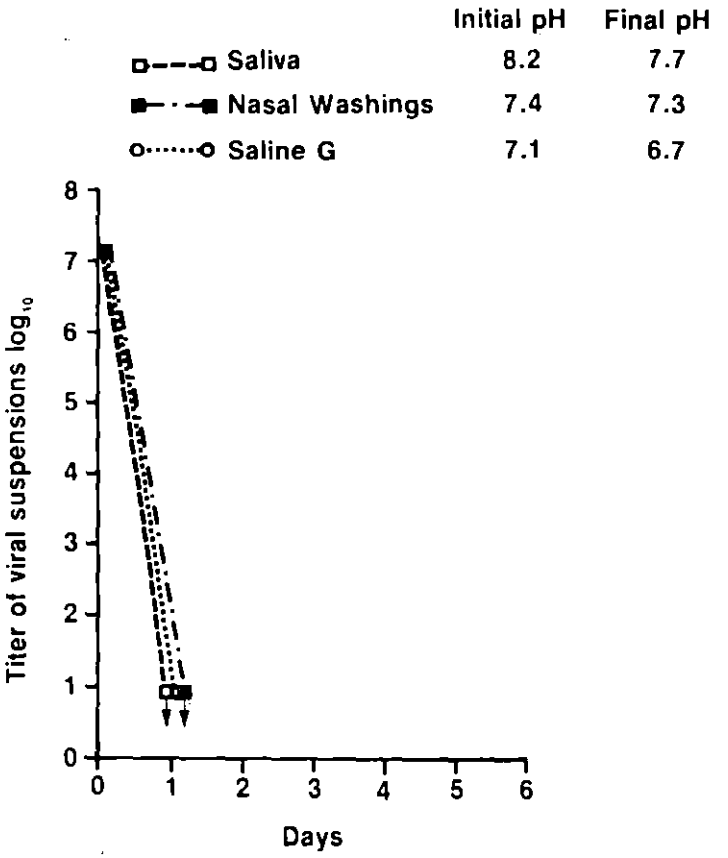

Figure 6. The effects of denim/diluents on the titers of pseudorabies virus

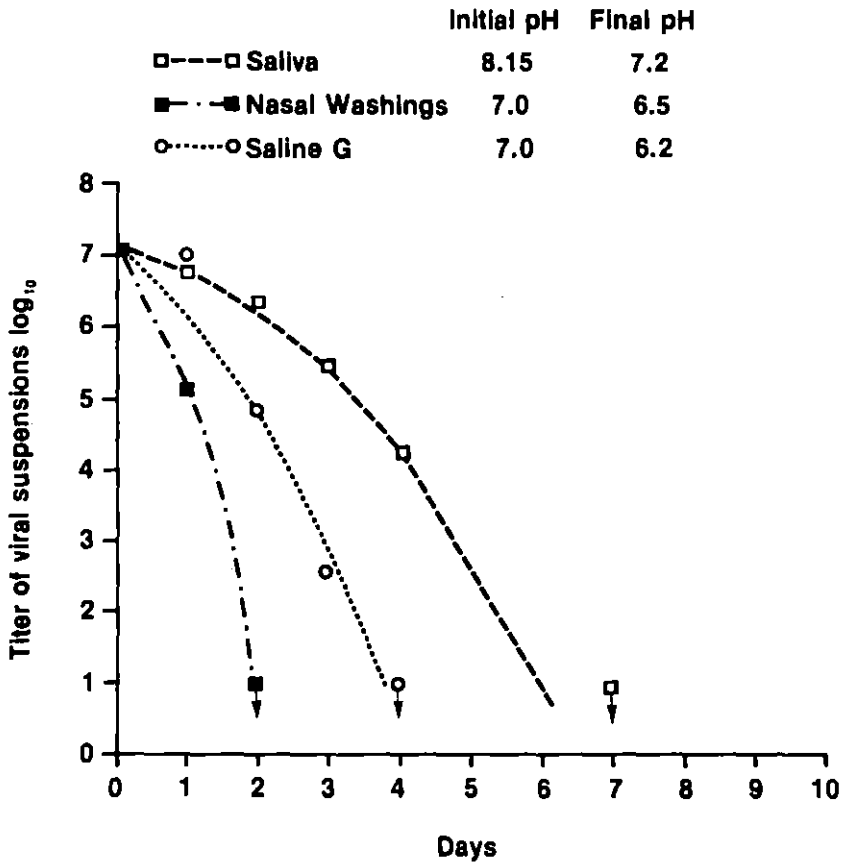

Figure 7. The effects of loam soil/diluents on the titers of pseudorabies virus

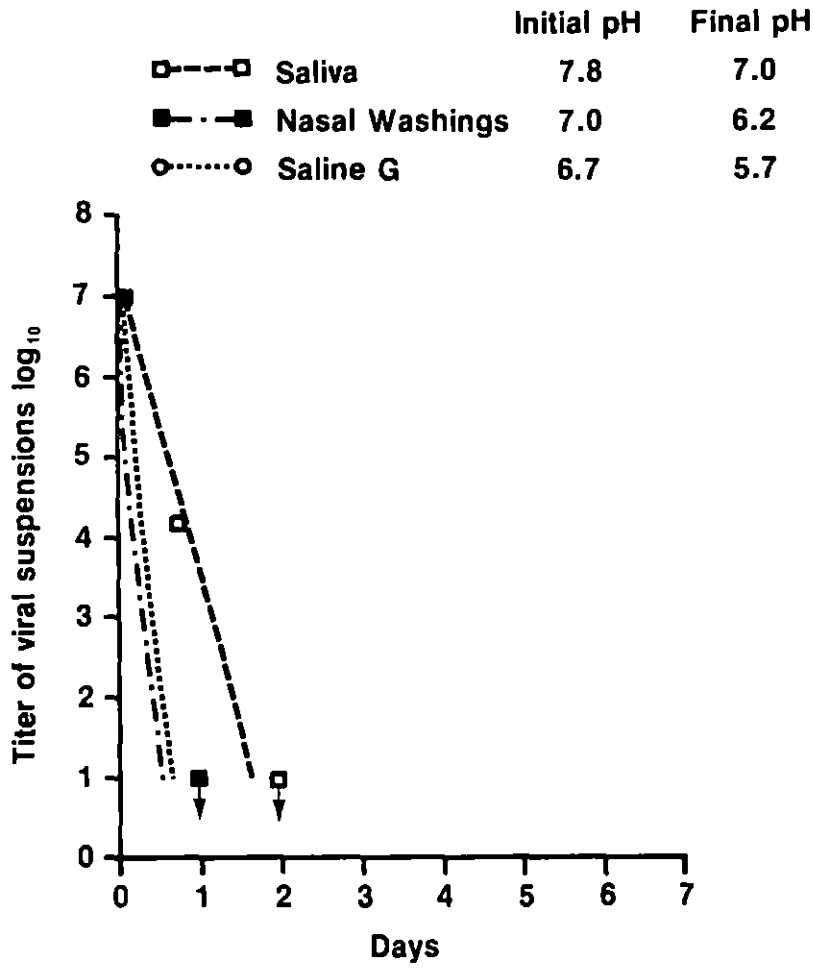

Figure 8. The effects of green grass/diluents on the titers of pseudorabies virus

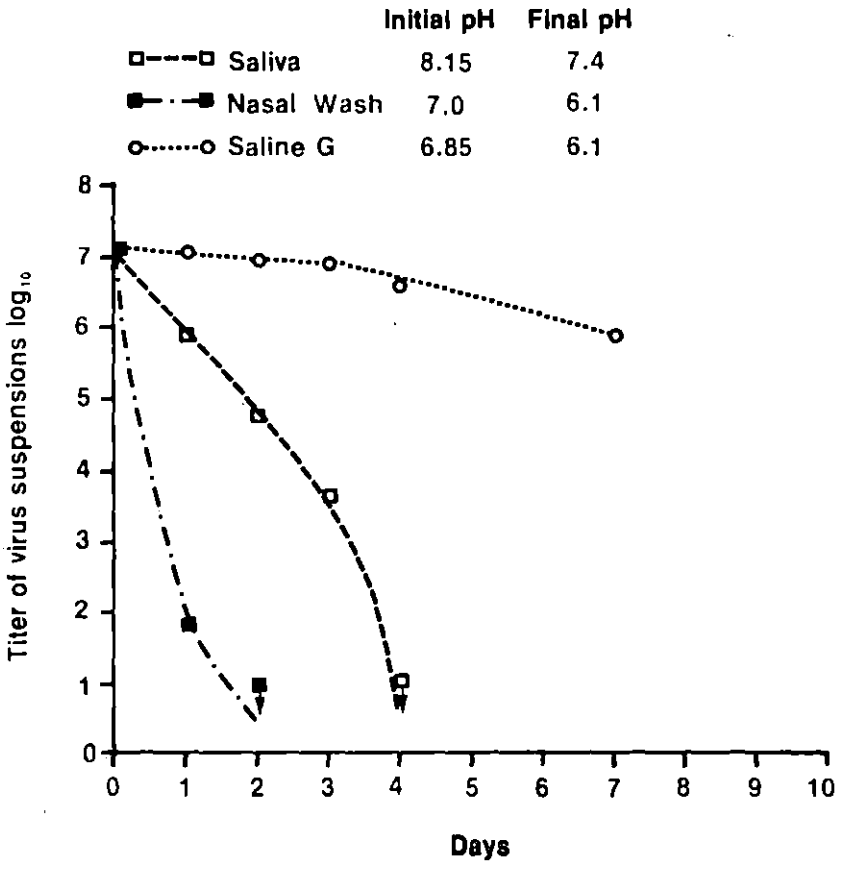

Figure 9. The effects of whole corn/diluents on the titers of pseudorabies virus

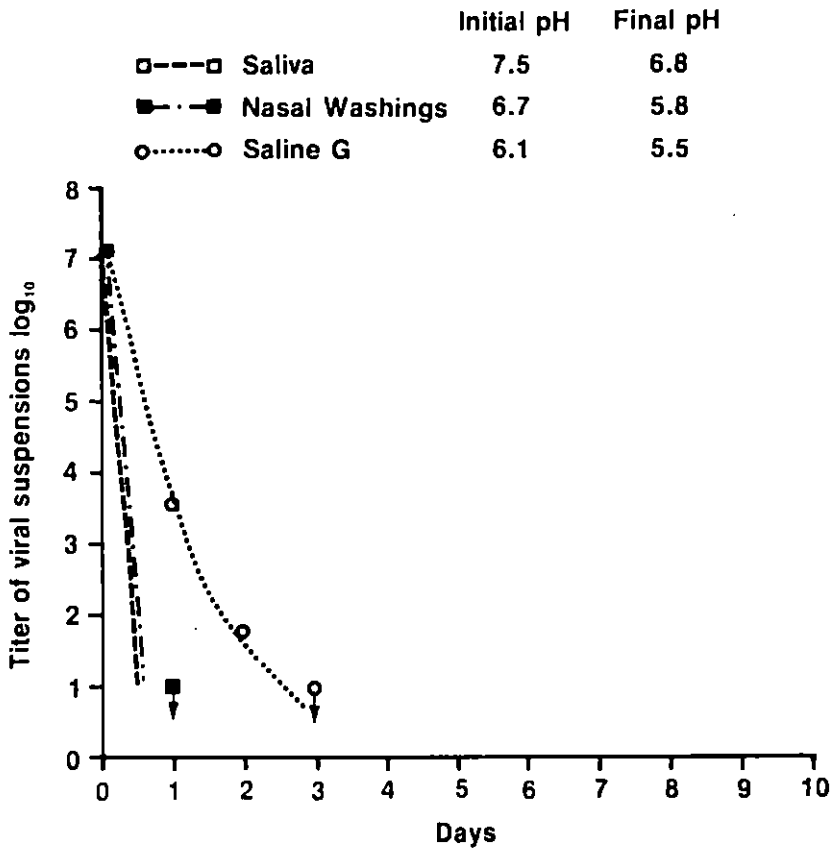

Figure 10. The effects of pelleted feed/diluents on the titers of pseudorabies virus

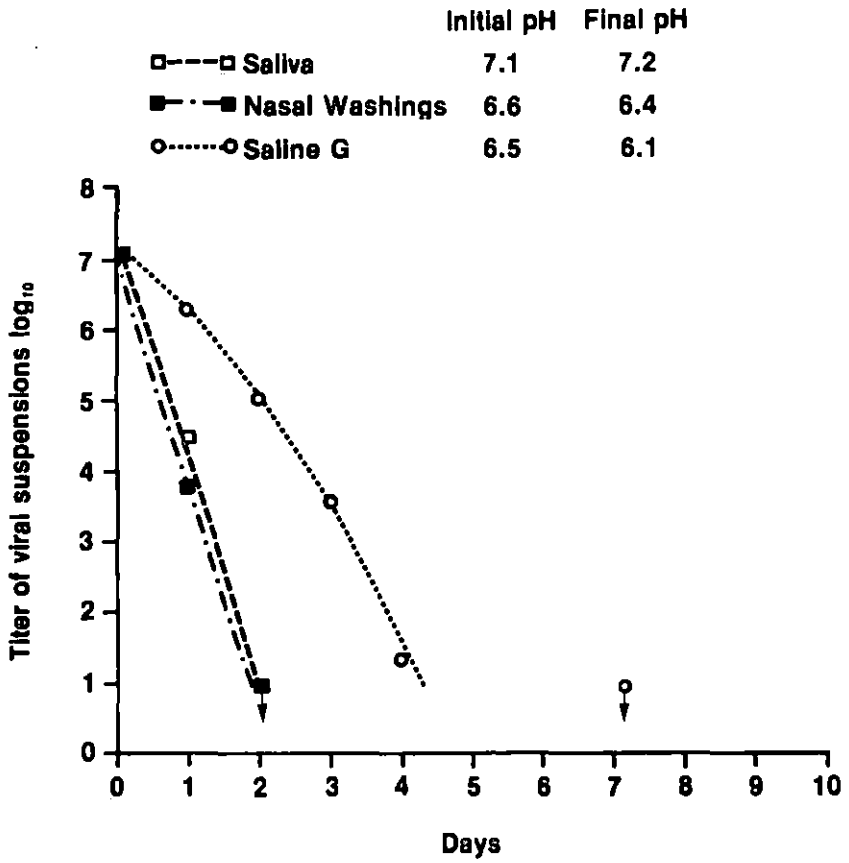

Figure 11. The effects of meat and bone meal/diluents on the titers of pseudorabies virus

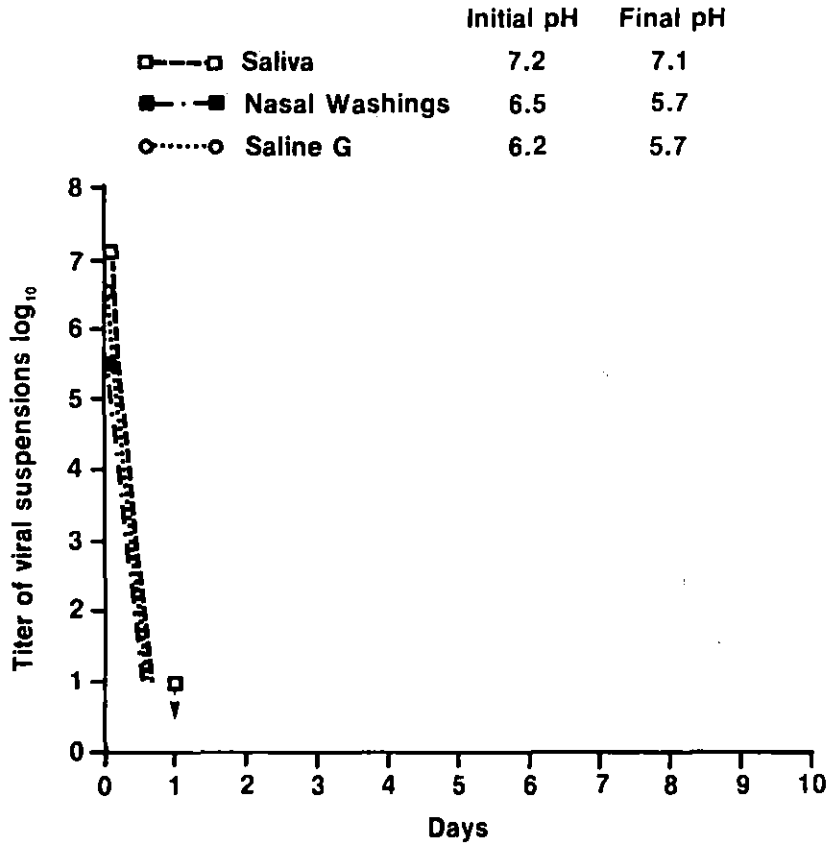

Figure 12. The effects of alfalfa/diluents on the titers of pseudorabies virus

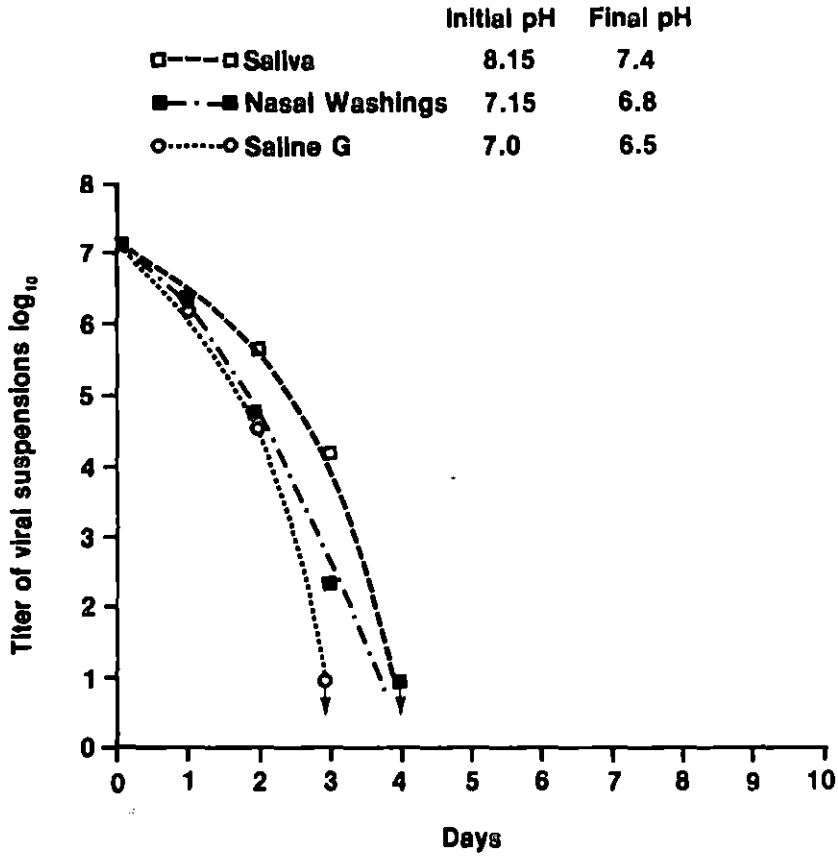

Figure 13. The effects of straw/diluents on the titers of pseudorabies virus

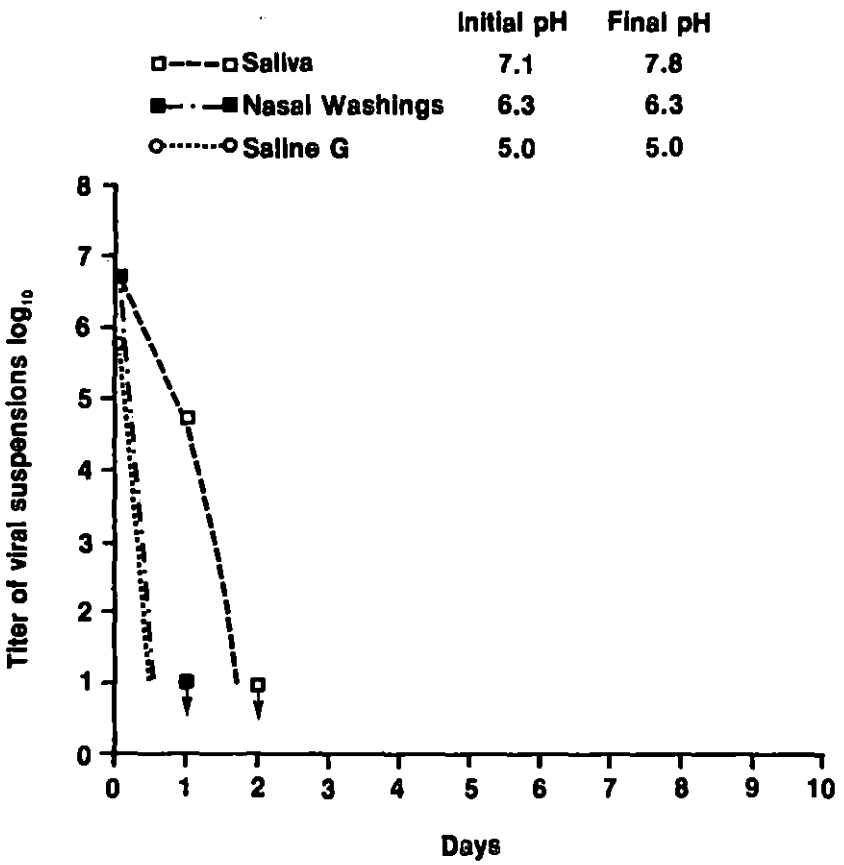

Figure 14. The effects of wood/diluents on the titers of pseudorabies virus

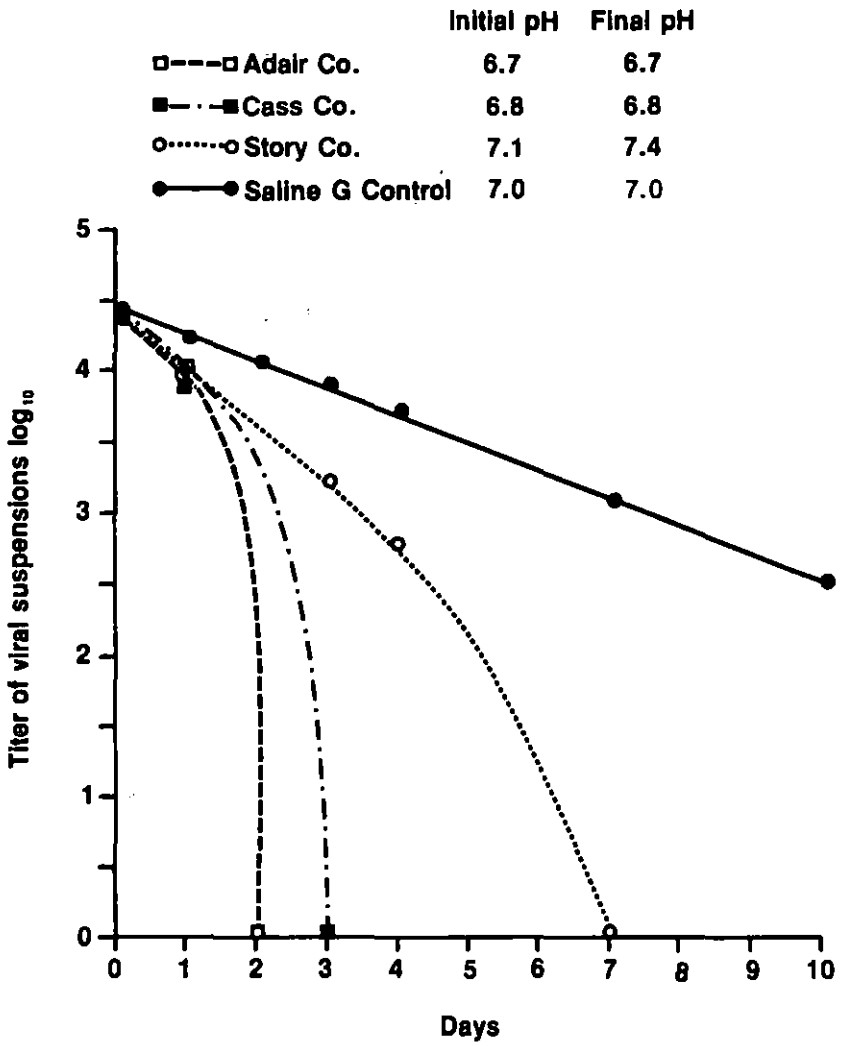

Figure 15. The effects of well water on the titers of pseudorabies virus

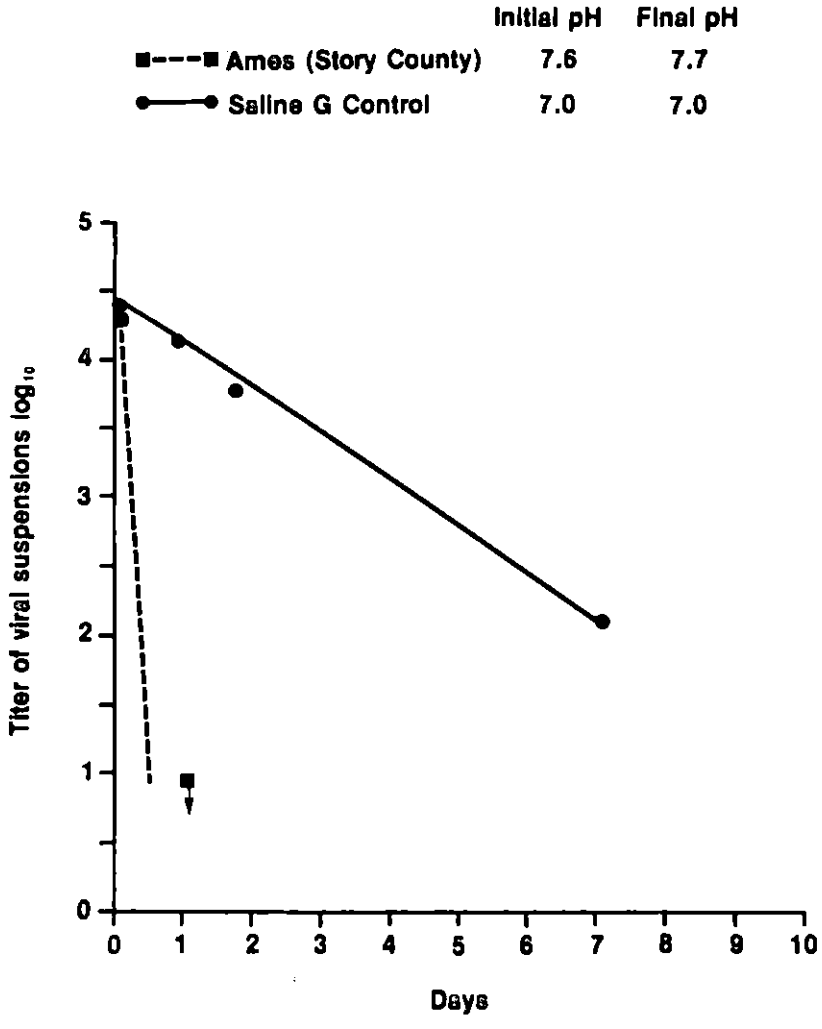

Figure 16. The effects of chlorinated water on the titers of pseudorabies virus

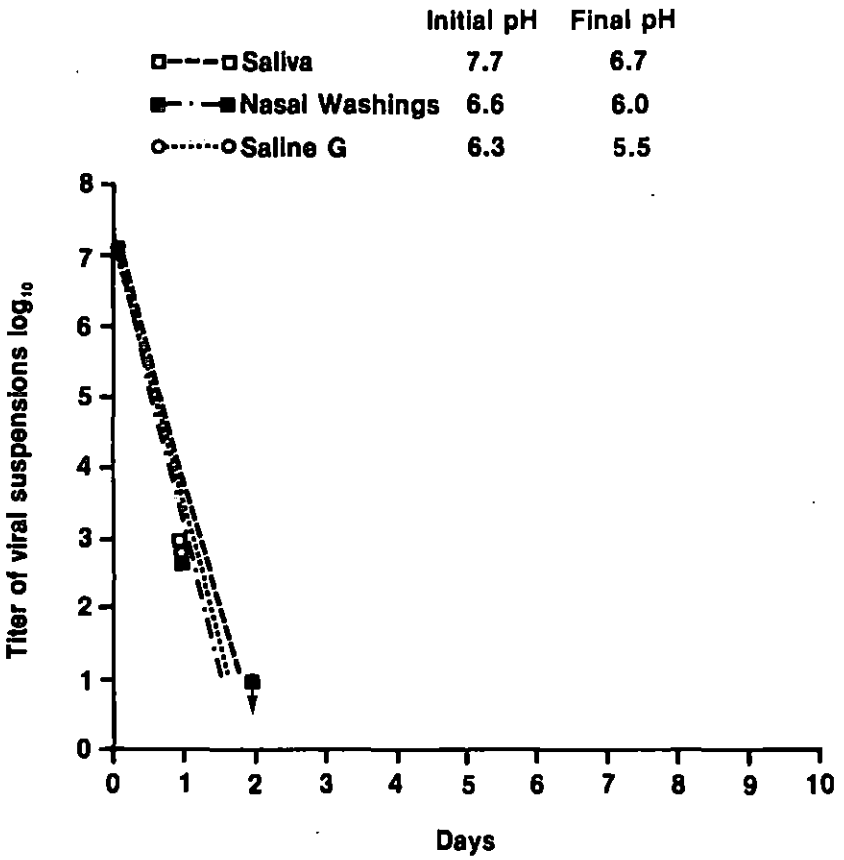

Figure 17. The effects of feces/diluents on the titers of pseudorabies virus

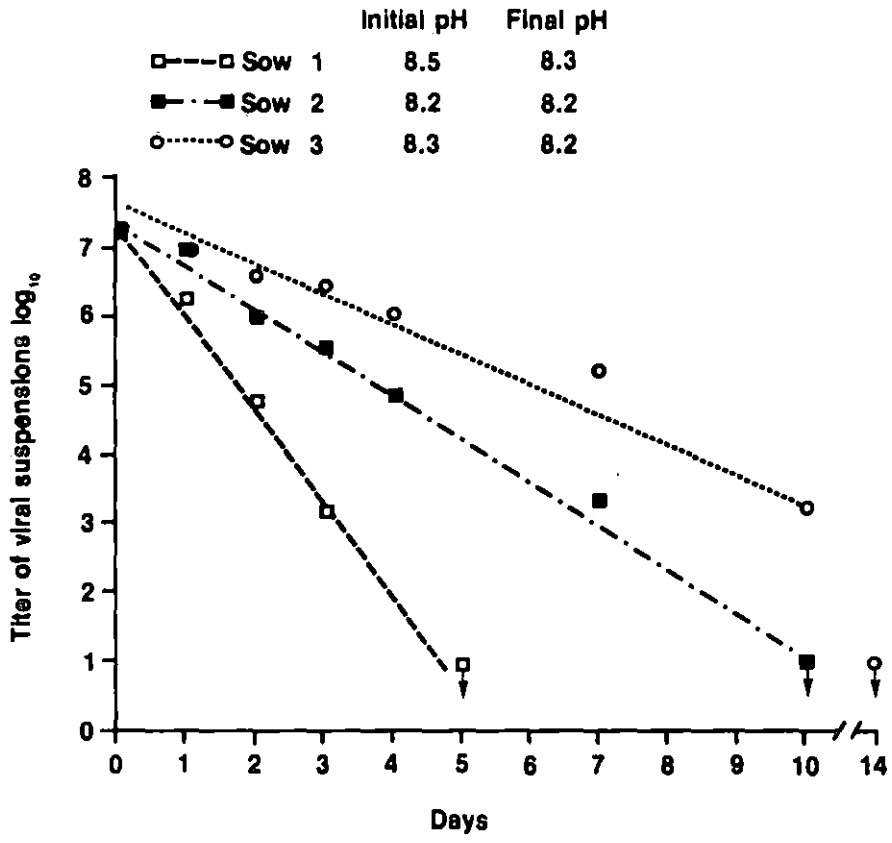

Figure 18. The effects of sow urine on the titers of pseudorabies virus

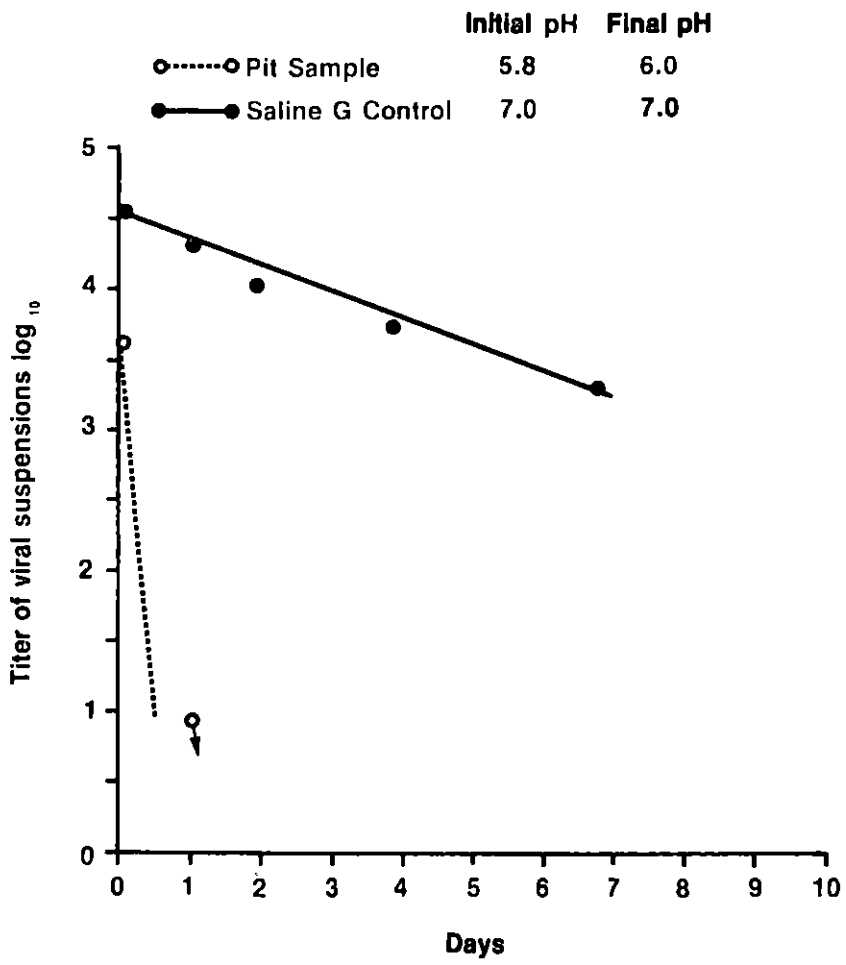

Figure 19. The effects of a swine pit on the titers of pseudorabies virus

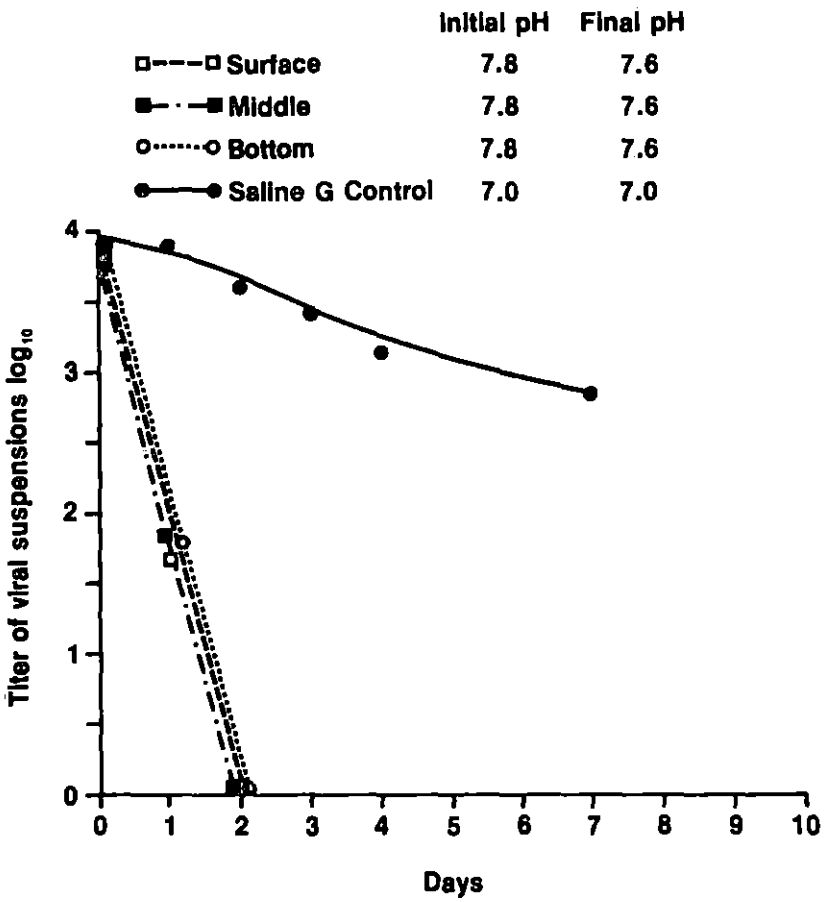

Figure 20. The effects of Adair County lagoon water on the titers of pseudorabies virus

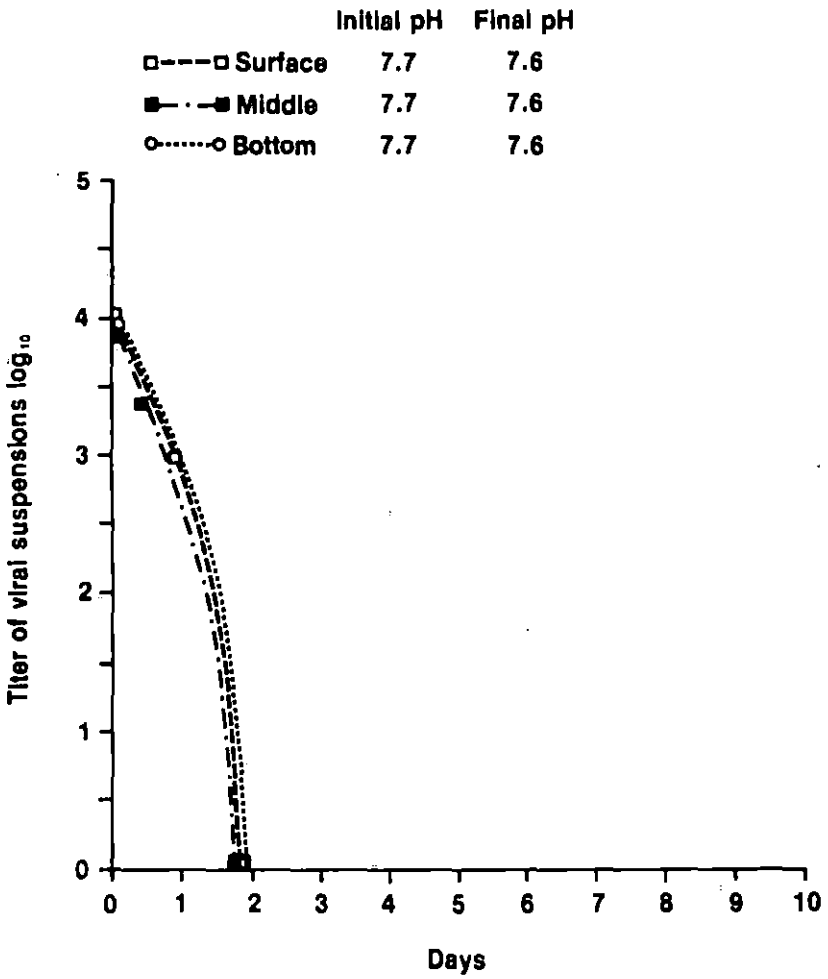

Figure 21. The effects of Cass County lagoon water on the titers of pseudorabies virus

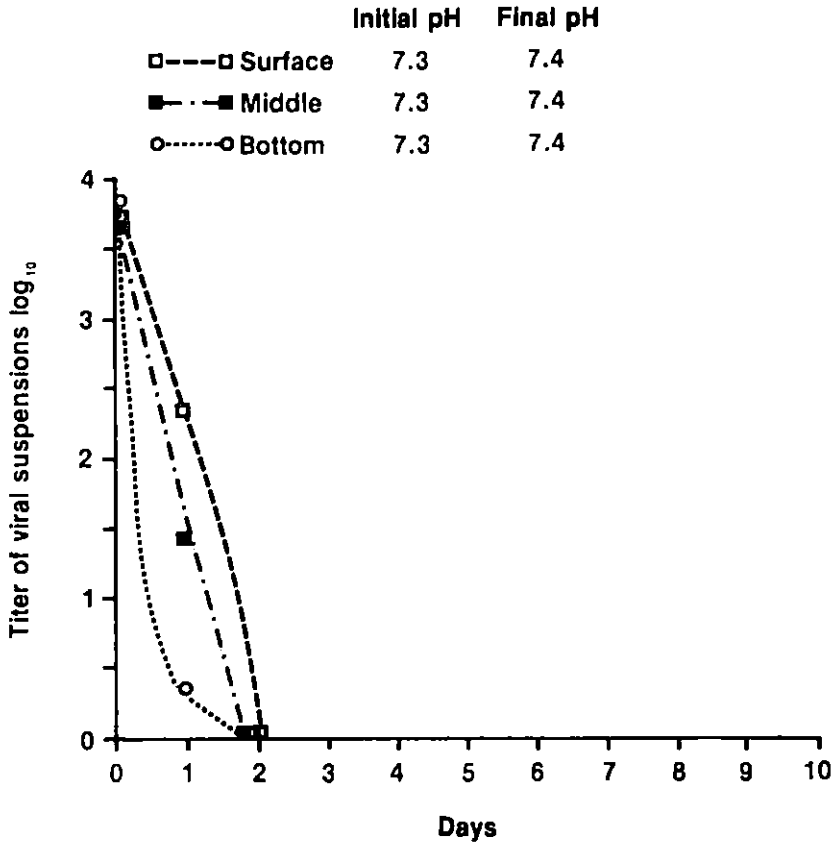

Figure 22. The effects of Story County lagoon water on the titers of pseudorabies virus