A study on the use of disinfectants against pseudorabies virus under selected environmental conditions

by

Mohammed Aboud Muhsin

A Thesis Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of MASTER OF SCIENCE

Department: Veterinary Microbiology and Preventive Medicine
Major: Veterinary Preventive Medicine

Iowa State University
Ames, Iowa

1982
**TABLE OF CONTENTS**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>iv</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>GENERAL FAMILY CHARACTERISTICS OF THE HERPETOVIRIDAE</td>
<td>3</td>
</tr>
<tr>
<td>Characteristics of Pseudorabies Virus</td>
<td>5</td>
</tr>
<tr>
<td>Pseudorabies Disease</td>
<td>6</td>
</tr>
<tr>
<td>EPIDEMIOLOGY OF PSEUDORABIES</td>
<td>9</td>
</tr>
<tr>
<td>ECONOMIC IMPACT OF PSEUDORABIES</td>
<td>15</td>
</tr>
<tr>
<td>PSEUDORABIES VIRUS SURVIVAL OUTSIDE THE LIVING HOST</td>
<td>18</td>
</tr>
<tr>
<td>DISINFECTANTS</td>
<td>24</td>
</tr>
<tr>
<td>Viral Disinfectants</td>
<td>26</td>
</tr>
<tr>
<td>Disinfectants in Use</td>
<td>27</td>
</tr>
<tr>
<td>Chlorhexidine</td>
<td>28</td>
</tr>
<tr>
<td>Benzyl Ammonium Chloride</td>
<td>31</td>
</tr>
<tr>
<td>Phenolic Compounds</td>
<td>37</td>
</tr>
<tr>
<td>Alkalies - Sodium Hydroxide</td>
<td>41</td>
</tr>
<tr>
<td>Chemical Inactivation of Herpesviruses</td>
<td>44</td>
</tr>
<tr>
<td>MATERIALS</td>
<td>47</td>
</tr>
<tr>
<td>Media</td>
<td>47</td>
</tr>
<tr>
<td>Diluents</td>
<td>49</td>
</tr>
<tr>
<td>Cell Cultures</td>
<td>52</td>
</tr>
<tr>
<td>Virus</td>
<td>53</td>
</tr>
<tr>
<td>Fomites</td>
<td>53</td>
</tr>
<tr>
<td>Disinfectants</td>
<td>54</td>
</tr>
</tbody>
</table>
### METHODS

<table>
<thead>
<tr>
<th>Topic</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Culture Preparations</td>
<td>64</td>
</tr>
<tr>
<td>Fomite Preparations</td>
<td>65</td>
</tr>
<tr>
<td>Disinfectant Preparations</td>
<td>65</td>
</tr>
<tr>
<td>Diluents</td>
<td>66</td>
</tr>
<tr>
<td>Virus</td>
<td>66</td>
</tr>
<tr>
<td>Virus Titration</td>
<td>66</td>
</tr>
<tr>
<td>Cell Culture Inoculation</td>
<td>67</td>
</tr>
<tr>
<td>Virus Assay Techniques</td>
<td>68</td>
</tr>
<tr>
<td>The Effects of Test Diluents</td>
<td>69</td>
</tr>
<tr>
<td>The Effect of the Combined Fomites and Diluents</td>
<td>69</td>
</tr>
<tr>
<td>The Effects of the Test Disinfectants</td>
<td>71</td>
</tr>
</tbody>
</table>

### RESULTS

<table>
<thead>
<tr>
<th>Topic</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effects of Environmental Factors on PRV Survival</td>
<td>74</td>
</tr>
<tr>
<td>Disinfectant Activity Against PRV</td>
<td>86</td>
</tr>
<tr>
<td>Effects of Diluents on Disinfectant Activity Against PRV, with and without Fomites</td>
<td>91</td>
</tr>
<tr>
<td>Effect of Fomites on Disinfectant Activity against PRV, in the Four Tested Diluents</td>
<td>101</td>
</tr>
<tr>
<td>Effect of Temperature with and without Fomites on Disinfectant Activity against PRV</td>
<td>102</td>
</tr>
</tbody>
</table>

### DISCUSSION

123

### CONCLUSIONS

131

### LITERATURE CITED

134

### ACKNOWLEDGMENTS

147
ABSTRACT

Disinfectants are used widely in farm practice as control and preventive measures against disease agents. This study was conducted to assess the activities of four categories of disinfectants in the presence of some natural environmental factors that were present in animal raising farms against pseudorabies virus (PRV) - a herpes virus. The four categories of disinfectants included chlorhexidine (Nolvasan\textsuperscript{R}), quaternary ammonium compound (Roccal\textsuperscript{R}), phenol derivatives (1 Stroke Environ\textsuperscript{R}), and alkali (lye). The environmental fomites that were tested were concrete, wood, plastic grate, soiled bedding and loam soil.

The eighth passage of S62/26 strain of PRV was prepared and stored at -90°C in 1 ml vials. The virus suspensions were thawed and diluted 1:10 in physiological saline solution (PSS) as suspending fluid. Tests were then performed using PSS, saline G, porcine saliva and porcine nasal washings as diluents with which the virus interacted in the presence of environmental fomites and disinfectants.

The disinfectants were initially diluted to 10X the manufacturers’ recommended concentrations for field use (RCFU) with distilled water, and from these dilutions, initial decimal dilutions at $10^{-1}$, $10^{-2}$ and $10^{-3}$ were made in PSS to give final test dilutions of 1X, 0.1, 0.01 and 0.001 from the
RCFU.

The environmental factors interacting with the virus in this study; i.e., fomites, disinfectants and diluents, were used in a ratio of 1:1:1:7 respectively, by adding 0.5g fomites, 0.5 ml of appropriate disinfectant solutions, and 0.5 ml virus solution to 3.5 ml of the test diluents for 5 or 30 min. reaction time at two temperatures, 4° and 25°C.

The activities of the disinfectants against PRV were studied in the presence of 7 environmental fomites in 4 diluents and at 2 environmental temperatures. Test conditions were rigorously controlled and all assays were performed by plaque reduction techniques in MDBK cells cultures.

Clean, weathered concrete exerted an alkaline pH effect (11.8 ± 0.4) in the presence of the 4 diluents tested, inhibiting the virus by itself and greatly enhancing the 4 disinfectants activity at both temperatures tested. Plastic grate showed a mild inhibitory effect on the virus, especially in saliva and this inhibitory effect improved the effectivity of the 4 test disinfectants as well. New wood had an inactivating effect on PRV by its acid pH (5.2 ± 0.4) in the presence of PSS, saline G and nasal washings. However, when disinfectants were applied, new wood protected the virus from their action, inhibiting by >90% the 4 disinfectants in these diluents. In the presence of saliva,
however, new wood lost its inactivating activity for the virus while still protecting it from the 4 disinfectants activities. Soiled bedding inhibited by >90% the activities of Nolvasan and Roccal, and unless maintained at RCFU levels, these two disinfectants were not found effective for soiled bedding disinfection. I Stroke Environ and sodium hydroxide were still effective if applied in concentrations not \(<\frac{1}{10}\) of their RCFU levels. Loam soil tied up Nolvasan and Roccal activities in the presence of the 4 diluents, and only I Stroke Environ and sodium hydroxide were effective if they were used in concentrations not \(<\frac{1}{10}\) of their RCFU.

Thus, concrete and plastic grate improved disinfectant activities while new wood, plastic grate and loam soil inhibited the 4 disinfectants. In the absence of interfering fomites, Nolvasan was more effective at 25°C than at 4°C, but no significant differences were noted in the activities of the other 3 disinfectants at either temperature. I Stroke Environ was more effective when used at 25°C than at 4°C to disinfect new wood. All 4 disinfectants were more effective at 4°C than at 25°C for disinfecting loam soil.
INTRODUCTION

The chain of transmission of any infectious disease, including pseudorabies, includes the presence of an infected host, escape of the infective agent from the host, the survival of the agent in the environment outside the host, the transport of the agent to a susceptible host, the entrance of the agent into this susceptible host, and the establishment of infection in this host. Efforts to prevent and to control infectious diseases are directed toward the links in this chain of transmission.

At critical points, attempts are made to break the chain by treating the infected host, by preventing escape of the infective agent from the host, by attacking its survival outside the host, by preventing its transport to and entry into a susceptible host and by preventing establishment of infection in the new host. Chemical, biological and environmental manipulations are most used in these attempts.

The goal of this study was to assist in interrupting the survival of pseudorabies virus in the environment. Experimental tests were directed toward the use of chemical disinfectants to prevent survival of pseudorabies virus outside the living host when suspended in body fluids and in contact with environmental fomites typical of the farm settings in which pseudorabies virus may be quite affected.
by the organic or inorganic materials in the environment into which its falls. The infective virus also escapes from the host with different types and quantities of organic materials, depending on its route of excretion. In this study, assessment was made as to how much and how long such organic materials and surrounding fomites could support the infective agent in the environment and how effective commonly used types of disinfectants could be in inactivating the virus in these environmental conditions to prevent its entry and infection into new susceptible hosts.

Pseudorabies virus, an enveloped herpes virus, causes heavy economic losses to the livestock industries of many countries. Under environmental conditions present in these countries, the virus has been found to survive in the environment for extended periods of time, often in spite of attempts to destroy it. Although the virus has been described as sensitive to many kinds of chemical disinfectants, experiences on infected farms have not been so uniformly successful in their use. The 4 disinfectants tested under a variety of environmental conditions in this study were representative of chlorhexidine, quaternary ammonium, phenolic and alkaline disinfectant compounds.
GENERAL FAMILY CHARACTERISTICS
OF THE HERPETOVIRIDAE

The Herpetoviridae is a family of viruses which includes pathogens for human beings as well as for a variety of wild and domestic animals. The virions have linear double stranded deoxyribonucleic acid (DNA) cores of 70 to 150 x 10^6 daltons molecular weight with a diameter of 75-77.5 nm enclosed in icosahedral capsids of 162 capsomers with a diameter of 90-105 nm. The nucleocapsids are coated with outer envelopes which render the precise definition of their diameter difficult, ranging between 150-200 nm. Within a population of virions, many particles do not possess envelopes and some are empty capsids (29, 47, 68).

The purified enveloped viral particles of herpes viruses, such as Herpes simplex, contain 25-30 virus specified proteins which constitute up to 70% of the virions (47). The DNA molecules which comprise approximately 7%, and 20-22% are host specified envelope phospholipids (47). Polyamines in small amounts are also found as spermine in the nucleocapsids and spermidin within the envelopes, as well as virus specific subunit glycoproteins which are also contained in the envelopes (42, 47).

Due to the presence of lipids in their envelopes, herpes
viruses are sensitive to chemical and physical agents (42, 68, 90), being readily inactivated by lipid solvents, variations of pH and by heat; also they are unstable at room temperature (42, 47, 90).

The Herpetoviridae family is divided into three subfamilies; Alphaherpesvirinae (Herpes simplex), Betaherpesvirinae (Cytomegalovirus) and Gammaherpesvirinae (Epstein-Barrvirus) antigens among members within the same genera which may be detected by several nonneutralizing immunological tests. Pseudorabies virus has been shown by gel immunodiffusion and by immunofluorescent techniques to have antigenic relations with Herpes simplex I and II, Herpes simiae (B-virus) and to lesser extents, with Marek's disease virus, turkey herpesvirus (44, 45, 66), and infectious bovine rhinotracheitis (IBR) virus (Reed, D. and K. Platt, personal communication, Department of Veterinary Microbiology and Preventive Medicine, ISU, Ames, Iowa, 1981).

The herpes viruses are characterized biologically by the formation of latent infections, which may be related to this intranuclear replication, though the mechanism of latency has not been well determined (29, 47). In laboratory cell cultures, they are characterized by cytopathic destruction of the infected cells and the formation of plaques or pocks which differ among virus strains (29, 47).
Characteristics of Pseudorabies Virus

Pseudorabies virus (PRV) was first reported by Schmiedhoffer in Germany in 1910 as the causative agent of the disease described in swine by Aujeszky in Hungary in 1902 and identified as Aujeszky's disease (AD) or as it had been called earlier in Europe, pseudorabies (PR) (68, 74).

PRV isolated from swine was found to be neutralized by serum from mad-itch convalescent cattle, and reciprocally mad-itch virus was shown to be neutralized by PR convalescent serum (105). These findings led to the conclusion that the causative agents of both diseases was one virus with two immunologically identical strains (106). In 1962, Andrews used the binomical name Herpesvirus suis for this virus, the only known herpesvirus to naturally affect swine (68).

A wide range of mammalian cell cultures have been used to cultivate or isolate PRV. PRV produces type A cowdry intranuclear inclusion bodies with plaque formation due to cytopathic effect (cpe) in cell cultures (47, 68). Characteristics of the cpe depends largely on the strain of the virus virus (96, 97). The correlation between the pathogenicity of field strains of PRV and their ability to cause cell fusion—syncytia formation was studied previously (18), and it was concluded that strains of PRV possessing high pathogenicity for swine and cattle will regularly be syncytia-forming. In
another study (99), five isolates from cattle were examined with respect to number of properties, including cpe and found to be nonsyncytia-forming, and concluded that they were attenuated. Golais and Sabo (50) studied as many as 10 virulent and 4 attenuated strains. Dow, in Ireland, also reported (37) that four strains existed: NIA1 (5 to 20 percent mortality), NIA2 (10 to 20 percent mortality), NIA3 (10 to 100 percent mortality) and NIA4 (no mortality, useful for making vaccine). Mayer and Skoda tested (87) two modified and four virulent strains of PRV for behavior at different temperatures.

The optimum pH and temperature stability were reported to be between pH 6 and 8; the greatest temperature stability of unfrozen virus was at 4°C and of frozen virus between -80°C and -90°C; at -13°C the virus was inactivated rapidly (34).

Pseudorabies Disease

Also called Aujeszky's disease, mad-itch, and infectious bulbar paralysis, PR is an acute infectious disease of swine and many other mammals. Under farm conditions, the virus has been found to cause disease in swine, cattle, sheep, dogs and cats (40, 62, 91). Although goats were excepted by some (65), they have been included in the lists of others (45). There is some doubt about natural susceptibility of the horse.
Deer, foxes, mink, badgers, coyotes, skunks, raccoons, opossum, rats, mice and rabbits have been listed as susceptible to natural infection (12, 54).

Swine are highly susceptible to PRV, developing peracute clinical disease with nearly 100% mortality during the first few weeks of life, but as they become older, they develop greater resistance to the clinical disease, especially to that caused by some strains (53, 58). It has been reported (120) that in some herds of swine the acute form of PR has never appeared and no visible signs of PR have been seen; yet, swine on these farms exhibit positive serum neutralization titers indicating the virus to be present on those farms, often in a latent form for a long period (2 to 3 years or more). PR infections in pigs involve the respiratory, nervous and reproductive systems; the principal manifestations are encephalitis and reproductive failures. Local pruritis may be present but is less marked in pigs than in most other species; occasionally infected cattle may not develop pruritis (15, 24). Domestic and wild animals of several species, other than swine, are highly susceptible to infection and develop severe clinical disease but the disease in them is usually of a noncontagious nature (55, 56). Unless carcasses of these animals are directly or indirectly consumed by pigs, infections in these species are likely to be dead end. All recovered
swine must be considered latently infected (22, 53, 54, 93, 120). While production of latent infection in organ cultures of species other than swine has been developed in the laboratory, the development of latent infection in occasional animals other than swine which recover from PR has not been determined (47).
EPIDEMIOLOGY OF PSEUDORABIES

It has been reported (57) that "Mad itch" in cattle had been recorded in the United States (U.S.) on September 10, 1813; that is about 90 years before identification of the disease in Hungary by Aujeszky. Also, it was reported (52) that despite the sickness and death caused to a wide range of animal species, not much attention was paid to the disease in the U.S. until the late months of 1962 when increasing losses in swine and domestic animals associated with them, including cattle, sheep, dogs and cats, began to be observed in the Midwest. The severity and spread of the disease in swine in the U.S. were similar to what had been observed for decades in Europe, including England, Ireland, Hungary, Netherlands, Germany, Denmark and Yugoslavia (90) (Kluge, John, Department of Veterinary Pathology, Iowa State University, Ames Iowa, personal communication). The disease has been recognized and reported (18, 46) from most European countries, Asia, North Africa, South America, as well as North America and recently New Zealand. In the U.S., Stewart (112), reporting on the widespread nature of the disease, noted that during the period from fiscal year 1966 through fiscal year 1972, the National Veterinary Services Laboratory, Animal and Plant Health Inspection Services (NVSL, APHIS), U.S. Department of Agriculture, had diagnosed
PR disease in pigs in eleven states. From the states mentioned during the "Pseudorabies Fact Finding Conference" April 4-5, 1977, in the U.S., major outbreaks were reported in Iowa, Illinois, South Dakota, Oklahoma, Missouri and Indiana.

Swine play the major role in contracting and spreading the disease as they have been found to be the only reservoir host (8, 10, 11, 12, 53, 55, 91, 118).

PR is usually contagious among swine only; other animals in general, contract the disease when they come into contact with pigs or their contaminated byproducts. Cattle, sheep, dogs and cats, as well as other domestic and wild animals have been found to become infected when they contacted infected swine (15, 21, 24, 63, 111, 114, 118, 120, 121). Trainer and Karstad (115) mention the possibility that wild mammals, such as foxes, skunks, muskrats, rabbits, raccoons, badgers, woodchucks, oppossum and deer could play a role in mechanical transmission of the virus through their carcasses, as most of these animals died before active shedding of virus could lead to transmission, but the virus was present in so many tissues of these animals that transmission could occur when their carcasses were consumed directly or indirectly by pigs. A study (123) on the possible transmission of PRV by raccoons concluded that although raccoons were susceptible
only to moderately large doses of virus (10⁴ to 10⁵ TCID₅₀) their carcasses could be a source of transmission. Recent studies at Purdue University have shown that PRV can be transmitted from pigs to raccoons and from raccoons to pigs through contact exposure, by eating at the feed trough and eating infected carcasses. Virus shed in saliva from infected raccoons was shown to contaminate feedstuffs which could then serve as a source of infection for ruminant animals (67). The same study also indicated that PRV may be shed by infected cattle and sheep. Animal species, other than swine, are not considered to be reservoir hosts, however, as there is no direct lateral transmission of the virus in these species (7, 8, 16, 17, 53). Generally, pigs play the major and frequently the only role in the transmission and spread of the disease, with the role of any other species being transient and over short distances only (53, 61, 81). In swine, vertical as well as coital transmission have been demonstrated (81). Swine display different clinical signs, depending upon their ages at the infection time, the virus strain involved and management of the herd (53, 81). The disease is usually fatal to swine only in their first few weeks of age (53). Infected adult swine may show mild clinical symptoms but the infection is usually unnoticed, and the animals become latent carriers without going through
any clinical phase (42, 46, 49, 81, 108, 120).

Contact transmission usually occurs by inhalation by susceptible pigs, directly into the moist nasal orifices, of virus exhaled or in oral secretions of clinically infected or carrier pigs, or indirectly from contaminated food or fomites on which virus has been deposited from the nasopharynx of clinically infected or carrier swine. The incidence of PR in swine has been reported to occur more frequently in large units than in smaller ones, due to increased contact between infected and susceptible hosts (1). The disease frequency has been found to increase in winter and early spring (69), which also is considered to be due to housing the animals in greater confinement with reduced air flow during the winter, resulting in increased respiratory contact. In 1972, Baskerville (6) reported that a minimum infective dose of virus of $10^{5}$ TCID$_{50}$ could initiate the disease but fever did not develop until the eighth day postinoculation. It was also reported (69) that specific-pathogen-free (SPF) pigs 36-days of age when inoculated with $10^{3}$ plaque-forming units (PFU)/ml of PRV, developed clinical signs within 48 to 72 hours, and six of fifteen died in the first week (5 to 8 days postinoculation).

During the clinical phase, the virus may be found to be shed through nasal and oral discharges (52, 76, 88),
especially saliva, from the first day of illness up to 17 days after onset (22, 76, 89). In addition to nasal and oral secretion of PRV, some workers have reported the isolation of the virus from urine, feces, milk and from boars at service (52, 75, 89). Others were not able to isolate PRV from urine or feces of affected animals (106), although the virus may be recovered from mucosa of the ileum and urinary bladder of such animals (13, 33, 85, 86, 107). Nikitin had been quoted (88) as having isolated the virus from urine for up to 186 days following clinical recovery. In infected boars, PRV has been recovered from prostatic and seminal vesicle fluids and from the prepuce (89).

Persistent carrier swine have been found to shed the virus for long periods following the first episode or time of infection (13, 47, 49, 79, 89, 124). In one study, it was reported (13) that the virus had been recovered from infected sows after 6 weeks to 13 months. In another study, PRV was shed by a sow that had been clinically infected 19 months earlier (33). In an additional study (114), the virus was isolated from a carrier sow 6 months after the initial episode. Cartwright in England, reported (Kluge, J, Department of Veterinary Pathology, Iowa State University, Ames, Iowa, personal communication), the isolation of PRV from a subclinically infected herd after more than
two years.

This carrier state or virus latency in infected pigs resembles infection by other herpesviruses in epizootiology, clinical signs, and pathological changes, including Herpesvirus B, Herpes simplex and infectious bovine rhinotrachitis viruses (46, 93, 109). Vaccination of swine with either attenuated live or inactivated vaccines has not eliminated the carrier state or prevented shedding of the virus by carrier pigs. However, vaccination has decreased the clinical signs and greatly reduced mortality in epidemic infection (1, 9, 21, 26; 84; 93, 114, 124).
ECONOMIC IMPACT OF PSEUDORABIES

Beran et al. (14) studied the economic impact of one epidemic in Iowa which extended for 16 months in three interrelated foci, to be about $0.5 million or around $28,912 per farm outbreak. Bedoya (10) reported the disease problem in Mexico when the first serious outbreak was recorded in swine in 1973; he noted that the disease killed 3500 piglets and in 1975 that it infected 3700 sows and killed 20,000 piglets. In the U.S., the disease is increasing in its cost impact; in 1974 there were 125 reported farm outbreaks, in 1975, 225 farm outbreaks, in 1976, 714 farm outbreaks, and in 1977, 1245 farm outbreaks. There were 535 farms quarantined in Iowa alone during the first 10 months in 1978. The estimated cost to the industry in 1977 was $21 to $25 million annually (March 22, 1978 APHIS news release). The USDA-APHIS (June 29, 1978) summarized the current threat of this disease to the U.S. swine industry, stating the following: "The number of outbreaks of PR is not only increasing at an alarming rate but financial losses to owners of infected herds are skyrocketing. Dollar losses in individual commercial swine herds have reached as high as $90,000 a year. One university researcher estimated producer losses to be between $21 million and $25 million per year. No estimate has been
made of damage done to the industry by losses in herds of seed stock producers, whose blood lines constantly add to the productivity of commercial herds. The spread of PR is expected to continue if unchecked by action programs. It has been estimated by the Swine Diseases staff that 60-70 percent of the nation's swine herds could be infected by 1982 if no control program is instituted. This figure was developed taking into consideration the rate of increase of seropositive swine surveyed in 1974 and 1977, and the infection rates of European nations where PR is endemic. Furthermore, since the disease will tend to infect a higher percent of the larger herds than of the smaller herds, 80 percent of our swine will originate in PR infected herds.

It was reported (1) that "after 1970, there was an increase in size of farms and also a marked increase in the incidence of A.D. The mean size of the farms rose from 20 sows in 1970 to 50 in 1978. In the beginning, it was the breeding herds that were the more seriously affected. Now, the major problems are in fattening herds; especially in the large ones with more than 1000 fatteners." He also added, that A.D. will reduce the mean daily growth rate by 30 to 50 gm and will increase the average mortality from 2% to 5% in fattening units.

The estimates of economic costs of PR to the swine
industry do not include the losses in animals other than swine, i.e., cattle, sheep, goats, dogs, and cats which come in contact with swine or of fur-bearing animals and other wild animals which succumb to the disease. As mentioned in the previous sections, swine play the major role in the dissemination of the disease and are the sources for the high losses to cattle and sheep farms (15, 24, 54, 74, 114, 119, 121).

It has been reported in the U.S. that the disease has reached the point where it represents a substantial loss to the swine industry and to the animal industry in general, and evidence indicates that the losses will continue to increase annually and that it will probably become more economically significant before effective methods of control are established (89, 124).

The greatest losses from PR are caused by the death of neonatal pigs (79). Mortality in baby pigs has varied from 1 or 2 pigs in a litter to 90% of the baby pigs in the herd at the time of the outbreak. Mortality in older animals is usually about 1-5%. In addition to losses by mortality and interruption of weight gain, PR has been shown to be a cause of reproductive problems in swine including abortions, mummified fetuses and stillborn pigs (22, 23, 72, 85).
Many research workers have studied, most in a limited manner, the survival of PRV outside the living host and have reported some effects of different environmental factors involved in enhancing the spread of the disease by protecting the virus excreted in the external environment by sick or symptomless carrier animals.

PRV was studied outside the living host at 25°C (44), and its survival was found to be dependent on the diluent and fomite combinations in which the virus was suspended or with which it came into contact. When nasal washings and saliva were used as diluents, PRV was inactivated within <2 days and <4 days, respectively, whereas in saline G and phosphate buffered saline solution (PBS), the virus survived for 58 days with minimal (<1 log) inactivation. When PRV was suspended in saline G and placed in contact with selected environmental fomites, the virus was found to survive for up to 7 days on steel and whole corn; for <7 days on plastic and rubber; for <5 days in meat and bone meal; for <4 days in soil, for <3 days on straw and pelleted food; and for <1 day on each of concrete, wood, alfalfa, greengrass and denim cloth. PRV suspended in nasal washings placed in contact with these fomites was
found to be inactivated in <4 days on straw; in <3 days on plastic; in <2 days on each of steel, rubber, soil, whole corn, meat and bone meal; and in <1 day on concrete, wood, alfalfa, pelleted feed, green grass and denim cloth. PRV suspended in porcine saliva as diluent and placed in contact with these fomites was inactivated in <7 days in soil; in <4 days on each of steel, concrete, whole corn, and straw, in <3 days on plastic, in <2 days on each of wood, meat and bone meal, green grass and rubber; and in <1 day on each of alfalfa, pelleted feed, and denim cloth. The same author found PRV to be inactivated within 2 to 7 days in nonchlorinated water and in <1 day in chlorinated water. PRV suspended in fresh swine feces or in effluent from swine pits or from lagoons was inactivated in <2 days, 1 day, or 2 days, respectively. In sow urine uncontaminated with feces, PRV remained at infective levels for 2-week-old pigs after 14 days. Other environmental factors, such as the effect of pH level and temperature on PRV, have been studied (34), and it has been found that the survival of PRV is strongly pH and temperature dependent. At 37°C, at an optimum pH level 6 to 8, the rate of inactivation was 0.6 log per day while at 4°C it was 0.04 per day. These authors found no apparent effect from fluctuation of the temperature between 6°C and 37°C upon the titer of the virus compared to a steady 37°C temperature. At a -13°C steady temperature,
the virus was rapidly inactivated at all pH levels. For long term storage, it was recommended that the pH should be adjusted between 6 and 7 and the temperature kept at -90°C (34). Drying on glass and on gelatin, as well as exposure to ultraviolet light, quickly inactivated the virus at all pH levels (34). PRV was also found to be stable at pH levels between 5 and 9 at 4°C and -60°C, and unstable at -15°C as well as when subjected to alternate freezing and thawing (125).

Other workers recorded 5 days survival of PRV in slurry in summer when the temperature was 12 to 18°C, pH level was 6.8 to 7.8 and humidity was 95 to 87% (98). PRV in fermented edible waste material has been reported to be inactivated at 20°C and 30°C within 24 hours, but the virus has survived for 48 hours at 10°C and for 96 hours at 5°C (122). On contaminated straw and feeding troughs, PRV has been reported to have survived more than 10 days at 24°C, for as long as 30 days at 18°C, and in carcasses or on contaminated wooden boards for 5 or 7 weeks, respectively (84). PRV was found to be inactivated in less than a month at -18°C but at 4°C survived for more than 6 months (84). The virus has been reported to have survived on hay, wood and food for up to 46 days (81). Others have reported (28, 46) that the virus was found to survive for 30 days in summer and 46 days in winter.

Viability of PRV was also studied (110) in fodder,
meadow hay, straw, oats, bran, potatoes, sacking, cord, boards and iron. In contact with these fomites, PRV becomes inactive in early spring between 32-46 days; in summer in 30 days (mean temperature 24.6°C); in the fall in 15-30 days (temperature 14.5°-18.5°) and in winter, between 40-46 days (mean temperature -8.4°C). These authors (110) also reported that environmental survival of the virus depended on climatic factors in various periods of the year, and that during sharp fluctuations in temperature, the virus become inactive earlier than during the absence of such fluctuations. Survival for 2-7 weeks in an infected environment and for up to 5 weeks in meat has been reported (22). PRV capability of surviving in infected premises and in dried tissues were reported (65) to be 2 months and several months, respectively. In the urine of pigs or sheep, PRV was found (3) to lose 3 logs of titer in 53 days at 4°C, and to lose the same in 18 days at room temperature.

Survival of PRV on wood alone and on wood soiled with manure was studied (117) under different seasonal temperatures. During the summer when the mean outdoor temperature was 19°C (66.2°F) and the mean temperature in closed swine units was 7.9°C (46.2°F), PRV survived on wood alone for 16 to 19 days, while on manure contaminated wood, it survived 13 to 16 days. In the fall when the mean outdoor
temperature was 10.5°C (50.9°F) and the mean temperature inside swine units was 4.7°C (40.4°F), the virus survived on the wood for 20 to 21 days while on the wood contaminated with manure it survived for 17 to 21 days. In winter and early spring when mean temperature were -9°C (15.8°F) outside and 4.5°C (40.1°F) inside swine units, the virus survived for 31 to 38 days on clean wood while on wood contaminated with manure, it survived for 22 to 25 days. In spring, when mean temperatures were 10°C (50°F) outside, and 15°C (59°F) inside swine units, the virus survived for 20 to 21 days on wood alone and on manure contaminated wood for 16 to 17 days. Generally, the virus survived approximately 3 days less on the wood contaminated with manure than on the wood alone. The humidity during these experiments was recorded to be between 77% in the fall and 86% in the spring. The same authors also studied the survival of PRV on the hides of infected animals and on experimentally infected hides found it to be 24-28 days in summer and 46-49 days in winter. The virus was reported (90) to have survived for 797 days in the fluid from a pulmonary edema lesion stored in a refrigerator. The same authors pointed out that PRV could survive for 30 to 50 minutes at temperatures ranging between 55-60°C, for 10 minutes at 70°C, for only 3 min. at 80°C, and that it was instantly inactivated.
at 100°C. It was also reported (84) that the virus was destroyed rapidly by boiling. In another study (50) on the effects of temperature and urea on virulent and attenuated strains of PRV, it was found that virulent strains were resistant to 53°C temperature while the attenuated strains were mostly sensitive.
DISINFECTANTS

A disinfectant is a chemical product that prevents infection by inactivation or inhibition of disease-producing organisms in the unanimate environment. It is desired that disinfectants function in the presence of extraneous organic matter and dense pathogenic microbial populations, regardless of the presence of saprophytic microorganisms, which may be found, that may or may not be killed (43, 59, 82, 100).

Desirable characteristics of good disinfectants have been reported to include: high germicidal efficacy with a wide spectrum of antimicrobial action lethal to bacteria, including bacterial spores, as well as to fungi, viruses and protozoa. A rapid lethal action in crevices and cavities and the penetration of organic material such as blood, sputum and feces are preferable. A disinfectant should be easily dissolved in or mix with water to form a stable liquid, non-corrosive to metal, and nondestructive to other materials (43, 59, 90, 93).

Disinfecting effectiveness of any disinfectants has been reported to depend upon several factors which might be related to the disinfectants such as solubility and stability in the medium and in microbe constituents, as well as affinity for bacterial cells, quality of ionization constant,
and mode of action. Effectivity may also be related to the microorganisms to be disinfected including susceptibility of the microbes, numbers and types of contaminants, presence of microbes as vegetative or spore forms, distribution of microbes in clumps or in even suspension. Finally, effectivity may be related to the general environment which might affect the disinfection process, including temperature at which the process occurs, time intervals of exposure to disinfectants, hydrogen ion concentration or level, pressure, especially in gaseous substances, presence of organic compounds or other chemicals that inactivate the disinfectant such as blood, sputum and feces, as well as electrolytes as sodium chloride, all of which have been reported to affect the disinfection process. Esthetic factors such as odor, color, or staining quality are sometimes determinants in the choice of disinfectants. Cost is often as important consideration especially for veterinary and farm applications (43, 51, 59, 90, 93).

The mechanisms of action of disinfectants have been reported to be through different means, including oxidation of microbial cells, hydrolysis, combination with microbial proteins to form salts, coagulation of proteins, modification of the permeability of microbial plasma membranes, inactivation of vital enzymes of microorganisms and
disruption of cell structures (5, 43, 64, 78, 82, 90, 95).

Viral Disinfectants

Dunham (38) mentioned that viruses can be inactivated by physical, chemical or biological agents. Physical agents include moist or dry heat and irradiation. Biological agents include body defense mechanisms.

Chemical disinfection of viruses has been found to be related directly to the type and concentration of the viral agents, to the presence of organic materials, to the time of exposure to the temperature at which the disinfectant is acting, and to the hydrogen ion concentration (2, 38, 64). Klein and Deforest (71) mentioned that the chemical composition of the outer coats of the test viruses determines their susceptibility to virucidal agents. The type of the virus affects the disinfecting activity of any disinfectant as in the case of enveloped viruses which contain lipid constituents in their envelopes (lipophilic viruses) that will be affected by lipid solvents and related materials (38, 64, 70). The same authors described hydrophilic unenveloped viruses which are not sensitive to, and sometimes are actually resistant to, lipophilic disinfectants unless used with other chemicals which change the pH or unless the concentration is increased to a higher level (5, 25, 38, 64, 71). The presence of organic materials, such as pus, blood,
serum or feces greatly inhibits the action of some disinfectants such as surface active agents and phenolic compounds (2, 5).

Disinfectants in Use

Kowalski and Mallmann (77) pointed out that through the survey they conducted among 100 veterinary practices in the state of Michigan, they found the most commonly used disinfectant to be quaternary ammonium compound followed by chlorhexidine. Many other workers as well as authors, have studied or mentioned the effectiveness of those disinfectants as well as of phenolic compounds and sodium hydroxide against herpes viruses (4, 5, 19, 25, 27, 39, 41, 64, 80, 86, 103, 116).

Thus, due to the above mentioned comments, and as this study dealt with one of the herpesviruses, PRV, a lipophilic virus; the following disinfectants were chosen for this study:

1. Chlorhexidine - Nolvasan<sup>R</sup>
2. Quaternary Ammonium Compound - Benzalkonium chloride - Roccal<sup>R</sup>
3. Phenolic compound - I Stroke Environ<sup>R</sup>
Chlorhexidine

Chlorhexidine is a synthetic, colorless, odorless and strongly basic compound; the chemical formula is: 1,1'-hexamethylene bis[5-(p-chlorphenyl)biguanide]. The chemical structure is:

\[
\text{Cl} \quad \text{C} \quad \text{C} \quad \text{C} \quad \text{C} \quad \text{C} \quad \text{Cl}
\]

\[
\text{NH} \quad \text{NH} \quad \text{NH} \quad \text{NH} \quad \text{NH} \quad \text{NH} \quad \text{NH}
\]

Chlorhexidine is a highly insoluble salt with a melting point of 134°. The diacetate salt solubility is far greater than the dihydrochloride salt (43, 64, 80).

Chlorhexidine has a strong alkaline reaction. It is only slightly soluble in water, so it is often formulated as a gluconate which has a good solubility in water. It is nontoxic and effective against a wide variety of Gram positive and Gram negative bacteria, and against other organisms (43, 64). It has been reported to have very little irritancy and low toxicity allowing it to be used on most sensitive mucosal surfaces (43).

Chlorhexidine has been reported to show its effect on bacteria on the cell membranes, inhibiting the adenosine triphosphate of the membrane by interfering with the net uptake of potassium ions by the intact cells. It has also been reported to disrupt the plasma membrane of
bacterial cells, even in the presence of blood (43, 59, 64, 92, 94). However, Lawrence (80) had reported
that chlorhexidine is reduced somewhat in antimicrobial
activity by certain organic substances, including milk, blood and serum.

It was predicted (73) that disinfectants with lipo-
philic properties might inhibit the infectivity of lipid
containing viruses as a result of damage of the exterior
of the virions causing interference with the early stage
of infection; i.e., attachment, penetration etc., and/or
as a result of injury to the viral nucleic acid, prevent
its complete functioning.

Chlorhexidine which has lipophilic properties (76),
has been described as the most widely used of the cationic
antiseptics (43) against lipophilic viruses (77). It has
been found to be effective against many members of the
herpesviruses; including PRV (25, 86); herpes simplex I and
herpes simplex II (104); feline viral rhinotracheitis virus
(100); equine infectious anemia (EIA) virus (102); and non-
herpesviruses such as transmissible gastroenteritis (TGE)
virus (25).

The chlorhexidine compound used in this study was
Nolvasan® (Fort Dodge Laboratories, Inc., Fort Dodge, Iowa).
The package describes the product as follows:
Active ingredient:
1,1'-Hexamethylene bis[5-(p-chlorophenyl)biguanide] diacetate = 2%
Inert ingredients = 98%

For use as a viricide and fungicide.

For use at the following concentrations:

1. "For disinfection of inanimate objects to aid in control of viral infection, 3 oz./gallon of clean water (23437.5 ppm),

2. For disinfection of veterinary or farm premises, 1 oz./gallon of clean water (7812.5 ppm),

3. For disinfection of all meat and food establishments, after removing or protecting the food carefully, use at a rate of 1 oz./gallon (7812.5 ppm) of clean water after thorough cleaning of the operational area."

Not effective against Pseudomonas aeruginosa or Gram positive pyogenic cocci on inanimate surfaces.

The manufacturers directions for use of Nolvasan in animal quarters are:

1. "Remove all animals and feeds from premises or from vehicles to be disinfected.

2. Remove all litter and manure from floors, walks, and any surface of the area to be disinfected.

3. Empty all feeding and watering appliances.

4. Saturate all surfaces with the recommended concentration of Nolvasan solution.

5. Ventilate premises or vehicles and do not return animals or use any equipment until the treated surfaces are dry.

6. All treated feeding and watering appliances must be scrubbed with detergents and rinsed with potable water prior to re-use."
The manufacturer cautions: "Keep out of reach of children. May be irritating to eyes or mucous membranes. May be harmful if swallowed. Avoid contamination of feed and feedstuffs. If in contact, flush thoroughly with water. Rinse empty containers thoroughly with water and discard."

Benzyl Ammonium Chloride

Also called Benzalkonium chloride, an alkyldimethyl-benzylammonium chloride. The structural configuration is:

\[
\begin{align*}
\text{CH}_3 & \quad \text{CH}_2 & \quad - & \quad \text{N}^+ \quad R \cdot \text{CH}_2^+ \\
\text{CH}_3
\end{align*}
\]

in which the R represents the alkyl radical ranging from \( \text{C}_8\text{H}_{17} \) to \( \text{C}_{18}\text{H}_{37} \). It is a cationic surface active quaternary ammonium compound (51, 59, 64, 95).

Benzalkonium chloride is a white amorphous powder readily soluble in water, forming a colorless and odorless stable, nonvolatile solution, which remains so under acidic, neutral or basic conditions. An increase in temperature and of the pH of the solution will increase its effectiveness (5, 78, 94).

The surfactants, both anionic and cationic preparations, have the ability to denature proteins (5, 59, 95). The antibacterial ammonium ions derive their unique biological
activity from the long aliphatic chain present in their structure which inhibits bacterial respiration and glycolysis, as well as inhibiting certain sensitive vital enzymes (5, 95). It has also been reported that at bactericidal and bacteriostatic concentrations, they decrease the surface tension and the permeability of the plasma membrane, thus, the organism loses its ability to remain in equilibrium with its environment (64, 92, 95). The positively charged cations of quaternary ammonium compounds combine with the negatively charged anions of soap, terminating the detergent activity of each other (5, 59, 64). Thus, skin should be washed thoroughly from soap before cationic disinfectants are used (59, 64).

Cationic disinfectants combine readily with proteins, fats and some phosphates (64), thus, they are of limited value in the presence of serum, blood, and other tissue debris, and are not suitable for disinfection of premises, as the large amount of organic debris present would immediately neutralize their antibacterial activity (2, 51, 59, 64, 78, 94).

It has been reported that cationic surfactants, in general, do not possess viricidal, fungicidal or spermicidal action (64). However, quaternary ammonium compounds have been found (71) to be effective against lipophilic viruses such as herpes simplex, vaccinia and Asian influenza.
viruses at a concentration of 1:10000 within 10 minutes, but not against hydrophilic viruses, polio type I, coxsackie B-1 and echo 6 even at a concentration of 1:10 for 24 hours. Activity against most lipid-containing animal viruses has been reported but not as a general viricide (77). Benzalkonium chloride at the rate of 1:1000 was found to be (38) effective against influenza and vaccinia viruses within 10 minutes; and against eastern equine encephalomyelitis virus with the presence of 1% tissue extract. In 1977, Knight (73) predicted that "viral infectivity may be lost by damage to the exterior of the virion in a manner that interferes with the early stages of infection (attachment, penetration, and so on) or by injury to the viral nucleic acid that prevents its complete functioning. Some treatments can cause both types of damage." Viruses with lipid material in their outer membranes permit penetration of the lipophilic cidal agents (5). Surfactant compounds with their chemical structure decrease the surface tension of an aqueous solution, and emulsification is augmented by altering the wettable surface and reducing the wetting angle (64). Benzalkonium chloride with its high affinity to combine with protein, fat or phosphorus (64); and its lipophilic properties (5, 71, 77), may function in these ways against herpes viruses.
Benzalkonium chloride has also been described (2) as effective against many types of viruses other than herpes viruses. The same authors reported (2) its inactivation of influenza type A, rabies, and Semliki Forest viruses. They also mentioned the sensitivity of measles and canine distemper viruses to the quaternary ammonium compounds, but vaccinia virus needed a higher concentration. The presence of serum inhibited the disinfectant action. It has also been mentioned that Zepheran chloride\textsuperscript{R} (a quaternary compound) was effective against adenovirus type 2, vaccinia and Asian influenza viruses as well as herpes simples (71). Quaternary ammonium compounds have also been found (25) effective against transmissible gastroenteritis (TGE) virus but not against porcine parovirus (PPV).

Benzalkonium chloride may be used to preserve the sterility of surgical instruments and rubber articles during storage at a concentration of 1:1000. A 10% aqueous solution of benzalkonium chloride is commonly used for general disinfection purposes (51, 59, 64, 78, 82).

The quaternary ammonium compound used in this study was Roccal (Winthrop, Sterling Drug Inc., New York, N.Y. 10016). The package describes the product as follows:
n-alkyl dimethyl benzyl ammonium chloride - 50% with (67% C₁₂; 25% C₁₄; 6% C₁₆; 1% C₈, C₁₀, C₁₈).

The manufacturer's recommended dilution for general disinfection of floors, walls, kennels, animal cages, furniture, boats and aprons was 1:625 (1600 ppm).

According to the manufacturer's information, Roccal activity has been demonstrated against canine distemper virus, canine herpes virus, and PRV at dilutions recommended for treatment of hard inanimate surfaces.

The manufacturer's directions for use of Roccal are:

1. For general disinfection of hard surfaces, "For floors, walls, operating tables, cages and furniture in animal hospitals, kennels, animal quarters and laboratories: clean thoroughly and rinse all surface areas before disinfecting. Then apply Roccal at a dilution of 1:400 (2500 ppm) by mop sponge or spray and let air dry. For surfaces difficult to clean and heavily contaminated areas, use Roccal at a dilution of 1:200 (5000 ppm)."

2. For disinfection of eating and drinking utensils for animals not considered as food sources for human consumption: "Scrape and prewash with warm water, then soak in Roccal at a dilution of 1:400 (2500 ppm) for at least 10 min."

"For the preparation of the recommended dilutions: For 1:400 (2500 ppm), add 10 ml (2 teaspoons) of Roccal to 1 gallon of water. For 1:200 (5000 ppm) add 20 ml (4 teaspoons) of Roccal to 1 gallon of water. Do not mix Roccal with soap or any anionic detergent."
The warnings of the manufacturer:

"Roccal is dangerous; keep out of reach of children. It is corrosive to tissues, causes eye damage and skin irritation. Do not get in eyes, on skin or on clothing. Wear goggles or face shields and rubber gloves when handling the concentrate. Harmful or fatal if swallowed. Avoid contamination of food."

For first aid in case of contact: "Immediately flush eyes or skin with plenty of water for at least 15 minutes. For eyes, call a physician. Remove and wash contaminated clothing before reuse."

"If swallowed, drink promptly a large quantity of milk, egg whites, gelatin solution or if these are not available, drink large quantities of water. Avoid alcohol. Call a physician immediately."

Note for physician: "Probable mucosal damage may contraindicate the use of gastric lavage. Measures against circulatory shock, respiratory depression and convulsions may be needed."
Phenolic Compounds

Phenol U.S.P. is one of the oldest antiseptics, having been introduced in surgery by Sir Joseph Lister in 1867. It is also called carbolic acid, due to its weakly acidic properties. It is a monohydroxybenzen (\( \text{OH} \)), obtained from coal tar (48, 64).

Phenol occurs as colorless to light pink crystals or crystalline masses that have a characteristic odor. It is soluble in water (1:15), very soluble in alcohol, glycerin, fixed and volatile oils as well as in petrolatum and liquid petrolatum (1:70). Its aqueous solution gradually darkens on exposure to air and light (48, 64).

In addition to its bactericidal activity, which is not very strong, phenol has a caustic, slightly anesthetic, and in general, protoplastic activity, being toxic to all types of cells. It causes protein precipitation under high concentration, whereas, at low concentration, it denatures protein without coagulation (48). It is also reported that under high concentration, phenol rapidly penetrates and ruptures cell walls, while in relatively low concentration, it permits the leakage of cell constituents, such as glutamic acid and other metabolites (5, 48). It has also been reported (59) that phenol is bacteriostatic in a concentration of approximately 0.2%, bactericidal above
1% and fungicidal above 1.3%.

Phenol has low affinity for protein, it does not combine firmly with the superficial proteins of cells but penetrates deeper into the tissues (59, 64, 95). Phenol action was described also as due to association with cellular lipids, because phenol has low water and high lipid solubility (5, 64, 95).

Thus, as a disinfectant with lipophilic properties, phenol might have the same mechanisms of action predicted (75) against lipid containing viruses, such as herpes viruses, by damaging the exterior of the virions and interfering with the early stages of infection; i.e., attachment and penetration. It was pointed out (5) that viruses with lipid materials in an outer membrane permit the penetration of the lipophilic cidal agents. Phenolic disinfectants may cause injury to the viral nucleic acid and prevent its complete functioning. Some treatments can cause both types of damage (73). The effectiveness of phenol against herpes simplex has been reported (71).

It has been reported that phenol is much more effective in aqueous solution than in glycerin or lipids, and it is relatively inactive when incorporated with soap (59). It has also been reported that the activity of phenol is not greatly reduced by the presence of organic matter, therefore, it is used mainly for disinfection of inanimate
objects and excreta (82, 94). The general antibacterial effects are irreversible by dilution with water, nor can bacteria affected by phenolic compounds acquire immunity to an inhibitory concentration (5).

Phenolic homologs are preparations with more lipophilic compounds to enhance the potential effectiveness of phenols. Many of the phenolic derivatives are more bactericidal than phenol itself, and the most important of these are the halogenated phenols, bisphenols, alkyl-substituted phenols and resorcinols (5, 48, 51, 59).

The phenolic disinfectant used in this study was I Stroke EnvironR (Vestal Laboratories, Division of Chemical Corporation, St. Louis, MO 63110). It is a mixture of phenolic compounds. The package describes the product as follows:

**Active ingredients:**

<table>
<thead>
<tr>
<th>Phenolic Compound</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-phenylphenol</td>
<td>10.0% (100,000 ppm)</td>
</tr>
<tr>
<td>O-benzyl-p-chlorophenol</td>
<td>8.5% (85000 ppm)</td>
</tr>
<tr>
<td>p-tertiary-amylphenol</td>
<td>2.0% (20000 ppm)</td>
</tr>
</tbody>
</table>

**Inert ingredients:**

<table>
<thead>
<tr>
<th>Inert Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>79.5% (795000 ppm)</td>
</tr>
</tbody>
</table>

Each of the active ingredients is more potent than phenol, but the toxicity and caustic actions are also greater (59, 82); n-amylphenol was found to gain increased effectiveness by increasing the molecular weight and it is
associated with decreasing the mammalian toxicity about 10 times less than phenol (5).

I Stroke Environ\textsuperscript{R} at a concentration of 1/256 (3906.25 ppm) at 23°C, was found to be effective against EIA virus (102); and feline calicivirus (FCV) (100). At a concentration of 1%, 0.75% and 0.5% (10,000 ppm, 7500 ppm and 5000 ppm), I Stroke Environ was found to be effective against African swine fever within one hour at room temperature when applied in a spray form (113). It has also been effectively used in a spray form against Marek's disease virus (MDV) at a concentration of 1/250 (4000 ppm) (27). The effectiveness of I Stroke Environ in the recommended concentration (RC) against PRV and TGEV but not PPV was also reported (25).

The manufacturer's directions for use of I Stroke Environ are: "For all general cleaning and disinfection, including tuberculous areas, use 1:256 solution (1/2 oz. per gallon of water) (3906.25 ppm). I Stroke Environ is harmless to fabrics, resilient flooring, tile, terrazzo, and painted or varnished surfaces. When cleaning and disinfecting food, animal or poultry facilities, first remove all livestock from the premises, cover or remove all feed and foodstuffs, and remove all dirt, litter and debris. Then thoroughly saturate all surfaces with I Stroke Environ use solution (Rinse all surfaces contacting food, and flush
feeders and waterers with potable water after disinfecting). Allow disinfected surfaces to dry before rehousing livestock."

The manufacturer warns: "Corrosive to tissues. The concentrate causes eye and skin damage. Harmful or fatal if swallowed. Do not get in eyes, on skin or on clothing. Wear goggles and rubber gloves when handling. Avoid contamination of food. Do not reuse empty container."

First aid: "In case of contact, immediately flush eyes or skin with plenty of water for at least 15 minutes. For eyes, call a physician. Remove and wash contaminated clothing before rinse. If swallowed, do not induce vomiting, drink large quantities of fluid and call a physician immediately."

Alkalies - Sodium Hydroxide

Since antiquity, alkalies have been reported to be used as disinfectants (64, 95).

Their mechanism of action is related to hydroxyl ion (OH⁻) concentration, and this depends on the degree of dissociation of the drugs in use (64, 90). A pH of greater than 9 has been reported to inhibit the growth of most bacteria (64). The vegetative forms of most bacteria will be killed in about 10 minutes in a 1 percent solution of sodium or potassium hydroxide, with Gram negative forms
more susceptible than Gram positive (95).

The effectiveness of alkalies against viruses has been reported by some authors. They have been used occasionally to disinfect excreta from patients with virus infections, especially poliomyelitis (95). At 5% concentration, sodium hydroxide inactivated EIA virus slowly (102). However, Aleutian disease virus was found to be inactivated within 10 minutes exposure to 0.5% as well as to 1% sodium hydroxide (103). In two different studies, Marek's disease virus was inactivated by 3% sodium hydroxide (66), as well as by 2%, 1% and 0.5% concentrations of sodium hydroxide, but at 0.25%, it was slightly less effective in the time limit of 10 minutes (27). In another study the effectiveness of 2% sodium hydroxide (pH 12.7) against swine vesicular disease virus in hard water as well as in hard water and swine feces was described (60). The 2% sodium hydroxide was also described to inactivate the Hong Kong strain of swine vesicular disease virus within 2 minutes (20). Foot and mouth disease virus was inactivated within less than 15 seconds by sodium hydroxide at pH 12.5 at 4° and 25°C (101). For 1, 10 and 30 minutes exposure at room temperature, 4° and 37°C, Talfan virus and adenos virus were inactivated by sodium hydroxide at a final dilution of 1% (w/v) (35).
Sodium hydroxide (Lye, Soda Lye), is widely used in disinfection. Lye contains 94% sodium hydroxide, and is commonly used at a concentration of 1.88% (18.800 ppm) NaOH which has been found to be effective against a wide range of pathogens (64, 90, 95). Potassium hydroxide and calcium oxide and hydroxide have been reported to be used for disinfection by many authors as well (64, 80, 95).

Many authors in different parts of the world have reported (64) to have mixed 2% Lye (1.88%, 18.800 ppm NaOH) with many other disinfectants to increase their effectiveness. Belak and Kisary mixed 1% sodium hydroxide, 8% sodium hypochlorite, and 3-5% formaldehyde to inactivate transmissible gastroenteritis virus within 15 minutes. Walter and Coffee used an aqueous alkaline solution of hydrated lime and sodium hydroxide for control of cryptococcus neoformans in pigeon coops (64). The same author reported that sodium hydroxide and phenol were used as disinfectant at temperatures below freezing, i.e., -5° to 0°C; and that 2% sodium hydroxide and 2% cresol were used to inactivate hog cholera virus in excreta. Sodium hydroxide and potassium permanganate were used against Talfan virus and porcine adenovirus type 2 (35). A calcium hypochlorite mixture containing 2%-3% chlorine, 2% formol, and 2% sodium hydroxide at 60° to 70°C, has been effectively
used for disinfection of transport vehicles (64).

The most commonly used alkalies which have been reported (95) are: NaOH, KOH, NaHCO₃, NH₃, Na₂CO₃, Na₃PO₄, Ba(OH)₂ and Na₂B₄O₇·10H₂O. Also mentioned (64), are lime (quick lime-calcium oxide), slaked lime (calcium hydroxide), and sulfurated lime solution.

A 2% solution of sodium hydroxide was used in this study.

Chemical Inactivation of Herpesviruses

The disinfectants used in this study were proven by many workers to be effective in inactivating some members of the herpesviruses.

Chlorhexidine (Nolvasan):

Pseudorabies virus (PRV) was found to be very sensitive to chlorhexidine (Nolvasan®) (25); within 5 minutes the virus was completely inactivated, at the recommended 1/128 manufacturer's concentration of disinfectant. Feline viral rhinotracheitis virus was also found (100) to be inactivated by this recommended concentration of chlorhexidine within 10 minutes exposure time. Herpesvirus hominis strains were inactivated completely within 90 minutes exposure time at the recommended final dilution (200 µg/ml) chlorhexidine gluconate in PBS (4).
Quaternary ammonium compound (Roccal)

Pseudorabies virus (PRV) was inactivated within 5 minutes exposure to Roccal-D at a concentration of 1/200 (25). Fowl laryngotracheitis and herpes simplex viruses were inactivated in 10 minutes exposure time at the recommended concentration of benzalkonium chloride (Roccal or Zephran chloride) at both a temperature of 30°C and room temperature (2). Marek's disease virus was inactivated within 10 minutes exposure to quaternary ammonium compounds (27). Only 0.13% or less infectious laryngotracheitis virus survived when exposed to quaternary ammonium compound diluted in PBS to the manufacturer's recommended dilution (39). Feline viral rhinotracheitis virus was also sensitive to the manufacturers recommended dilution of quaternary ammonium compound and was inactivated within 10 minutes exposure (100).

Synthetic phenol compound (I Stroke Environ)

Pseudorabies virus was inactivated completely at the recommended dilution (1/256) of I Stroke Environ, a synthetic phenol compound, within 5 minutes (25). Marek's disease virus was also inactivated by the same preparation at a dilution of 1:250 within 10 minutes (27). Feline viral rhinotracheitis virus was inactivated by a 1/256 concentration of I Stroke Environ within a 10 minutes exposure.
time (100).

**Sodium hydroxide (Lye)**

Pseudorabies virus was inactivated by 5% sodium hydroxide within 5 minutes (25). Marek's disease virus was inactivated within 10 minutes by 2% sodium hydroxide (27); however, in another study, 3% sodium hydroxide was found to be ineffective in inactivating MDV (66). Infectious bovine rhinotracheitis virus was found to be sensitive to 2% sodium hydroxide (41, 83).
MATERIALS

The basic requirements for virus cultivation, propagation and identification, as well as detection of the survival by plague assay techniques, depends mainly on the media and cell culture systems in use. The media, cell cultures, diluents, virus, fomites and disinfectants used in this study were the following:

Media

The propagation and cultivation media prepared in the laboratory and used in this research work had been used in related research studies (32, 44).

Minimum Essential Medium (MEM)

Minimum Essential Medium (MEM) was prepared by dissolving gently at room temperature (15-30°), dry packs of powdered media (Grand Island Biological Company, Grand Island, New York) (Table 1), in deionized triple distilled water. The appropriate salts and nonessential amino acids were contained in the prepared media packs but not sodium bicarbonate. After the dry media were dissolved, this was added at a rate of 2.2 gm of sodium bicarbonate per liter. Then, the pH was adjusted to 0.2-0.3 units lower than the desired level of 7.0 (32) using 1N sodium hydroxide or 1N hydrochloric acid. The media were then sterilized
by filtration through methyl cellulose membrane filters (Millipore Corporation, Bedford, Massachusetts), of 200 nm pore diameter which raised the final pH to 7.0.

To control any contamination that might result in bacterial or fungal growth in the media or cell cultures, antibiotics were added as shown in Table 2. The final solutions were checked by plate inoculation for bacterial sterility.

Minimum Essential Medium (MEM), prepared in single and double strength, was stored at 4°C, from which the following were prepared.

**Growth Medium (GM)**

Growth Medium (GM) was prepared from single strength MEM by adding 10% fetal bovine serum (FBS) (Sterile Systems Inc., Logan, Utah), to the required quantity of MEM and also antibiotics if not added to the stock.

**Maintenance Medium (MM)**

Maintenance Medium (MM) was also prepared from the single strength MEM by the addition of 2% (FBS) to the required quantity of MEM, as well as antibiotics if not added to the stock.
Overlay Medium

Overlay Medium was prepared from double strength MEM with an equal quantity of gum tragacanth solution (described below) and 2% FBS, as well as antibiotics if not added to the stock MEM.

Gum Tragacanth Solution

Gum Tragacanth Solution was prepared by adding slowly 1.6 grams of gum tragacanth (Fisher Scientific Company, Pittsburgh, PA) to 100 ml of warm triple distilled water with agitation, and sterilized by autoclaving for 30 minutes at 121°C.

Diluents

The diluents used throughout the different experiments in this study (listed in Table 6-B) included the following:

Saline G

Saline G constituted the main virus diluent for comparative tests in this study. It had been used previously (13, 32, 33, 44). Table 3 shows the complete formula of saline G. The ingredients were dissolved gently at room temperature; pH was adjusted to 0.2-0.3 higher than the required level and sterilization was performed by filtration.
through a 200 nm pore diameter filter (Millipore Corporation, Bedford, Massachusetts). The pH was then corrected by adding 0.1N sodium hydroxide or 0.1N hydrochloric acid to reach the required pH 7 before using the solution in this study.

**Physiological Saline Solution (PSS)**

Physiological Saline Solution (PSS) was prepared by dissolving 8.5 grams of sodium chloride in one liter of distilled water. Sterilization was by autoclaving for 15 minutes at 121°C. The pH was then adjusted to between 6.9-7.1 with 0.1N sodium hydroxide or 0.1N hydrochloric acid.

**Phosphate Buffer Solution (PBS)**

Phosphate Buffer Solution (PBS) was used in some experiments in this study. Table 4 shows the full formula. The materials were dissolved in triple distilled water and sterilized by autoclaving for 20 minutes at 121°C. PBS was prepared in single strength and ten times strength.

**Distilled water**

Distilled water was used to dilute the disinfectants to double or ten times the strength (2X or 10X) of the manufacturers' recommended concentration, and was used in the preparation of different other diluents in this study.
as well.

**Trypsin-Versene solution**

Trypsin-Versene solution was used to remove the cell monolayers from the substrate. The complete formula is shown in Table 5. The solution was prepared by mixing the constituents other than trypsin gently in 180 ml of triple distilled water, heating the mixture to 37°C then adding the trypsin over approximately one hour with continuous mixing to dissolve completely. Triple distilled water was then added to make 200 ml and the solution was sterilized by filtration through a methyl cellulose filter (Millipore Corporation, Bedford, Massachusetts), of 200 nm pore diameter. This resulted in ten times the required concentration of trypsin-versine solution and it was diluted 1:10 in triple distilled water before use.

**Saliva**

Saliva was collected from 12, 3-4-week-old pigs tested free of PR. The pigs had been injected with one injection of 1% Pilocarpine hydrochloride intramuscularly (IM) each. The drooling saliva was collected in clean sterile beakers and stored at 4°C overnight to allow settling down of all debris and foreign ingredients suspended in the saliva. On the next day, the clear supernatant portion was aspirated and poured into sterile 50 ml
bottles and stored at -20°C.

Nasal washings

Nasal washings were collected from the nostrils of pigs tested free of PR, refrigerated and cleared of any suspended material that might be present, placed in bottles of 50 ml each and stored until used at -20°C.

Cell Cultures

The cell line used in this study was of Madin-Darby bovine kidney (MDBK) cells, the true passage level of which is not known. The same cell line was used in other studies in this laboratory (13, 32, 33, 34, 44).

Single-use plastic 250 ml tissue culture flasks (Corning Glass Works, Corning, New York), were used to grow the cell monolayers, using GM with antibiotics. The cultures were incubated at 37°C in a humid chamber with 5% carbon dioxide atmosphere with the screw caps loosened slightly to allow gaseous exchange.

After the cells had grown into full confluent monolayers, usually within 48-72 hours, the GM was poured off and the cells were removed from the flasks using trypsin-versene solution. Suspended cells were passaged into new flasks for another passage or into single-use eight-well plastic plates (Luxplate, Lux Scientific Corporation,
Newbury Park, California), for experimental use. They were incubated in the same environment mentioned above.

Virus

An eighth laboratory passage of PRV "Iowa strain" or "Wilson strain" S62/26 which was collected by G. W. Beran (Department of Veterinary Microbiology and Preventive Medicine, College of Veterinary Medicine, Iowa State University, Ames, Iowa) in 1974, and which was subjectively described as moderately virulent, was used throughout this study. The virus passage techniques were described by Davies (32) and the aliquots were separated in vials of 1, 3 and 5 mls each and stored at -90°C.

Fomites

The fomites included in this study are listed in Table 6a. They were selected as the most probable materials that may contribute to the transmission of the virus from infected to susceptible animals through the environment, and which may be chemically disinfected without causing any harm to the animals. PRV had been found to survive for variable periods on the same fomites by Freund (44) as well as other investigators discussed in the literature review.
Disinfectants

The disinfectants listed in Table 7 were chosen for this study primarily because they represented the active ingredients of most commercially available disinfectants. Secondly, they are widely used in laboratories and veterinary clinics as well as on farms. Thirdly, their effectiveness against enveloped viruses in general, and especially herpes viruses, has been well-documented.

The concentrations of the disinfectants used in these experiments were serial dilutions from the manufacturer's recommended concentrations for field use (FCFU). The disinfectants were first diluted to 10X or 2X the RCFU with distilled water and then to the test dilutions with the virus-diluent-fomite mixture used in the tests.
Table 1. Minimum Essential Medium (MEM) composition

<table>
<thead>
<tr>
<th>Component</th>
<th>Ingredients</th>
<th>Weight (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>a. Salts and sugar components</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Earle's salts</td>
<td>Calcium chloride anhyd. (CaCl₂)</td>
<td>200.00</td>
</tr>
<tr>
<td></td>
<td>Potassium chloride (KCl)</td>
<td>400.00</td>
</tr>
<tr>
<td></td>
<td>Magnesium sulfate anhyd. (MgSO₄)</td>
<td>97.67</td>
</tr>
<tr>
<td></td>
<td>Sodium chloride (NaCl)</td>
<td>6800.00</td>
</tr>
<tr>
<td></td>
<td>Sodium phosphate, Dihydrogen (monobasic) (NaH₂PO₄·H₂O)</td>
<td>140.00</td>
</tr>
<tr>
<td>Sugar</td>
<td>Glucose</td>
<td>1000.00</td>
</tr>
<tr>
<td>Phenol red</td>
<td></td>
<td>10.00</td>
</tr>
<tr>
<td><strong>b. Amino acids and vitamins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Alanine</td>
<td>8.90</td>
<td></td>
</tr>
<tr>
<td>L-Arginine·HCl</td>
<td>126.00</td>
<td></td>
</tr>
<tr>
<td>L-Asparagine·H₂O</td>
<td>15.00</td>
<td></td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>13.30</td>
<td></td>
</tr>
<tr>
<td>L-Cystine·2HCl</td>
<td>31.29</td>
<td></td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>14.70</td>
<td></td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>292.00</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>7.50</td>
<td></td>
</tr>
<tr>
<td>L-Histidine HCl·H₂O</td>
<td>42.00</td>
<td></td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>52.00</td>
<td></td>
</tr>
<tr>
<td>L-Leucine</td>
<td>52.00</td>
<td></td>
</tr>
<tr>
<td>L-Lysine HCl</td>
<td>72.00</td>
<td></td>
</tr>
<tr>
<td>L-Methionine</td>
<td>15.00</td>
<td></td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>32.00</td>
<td></td>
</tr>
<tr>
<td>L-Proline</td>
<td>11.50</td>
<td></td>
</tr>
<tr>
<td>L-Serine</td>
<td>10.50</td>
<td></td>
</tr>
<tr>
<td>L-Threonine</td>
<td>48.00</td>
<td></td>
</tr>
<tr>
<td>L-Tryptophane</td>
<td>10.00</td>
<td></td>
</tr>
<tr>
<td>L-Tyrosine (Disodium salt)</td>
<td>52.10</td>
<td></td>
</tr>
<tr>
<td>L-Valine</td>
<td>46.00</td>
<td></td>
</tr>
</tbody>
</table>

*aGibco, Grand Island Biological Company, Grand Island, New York 14072. Available in appropriate packs of pre-mixed media along with L-glutamine, nonessential amino acids and some vitamins (Table 1-b) but without sodium bicarbonate.*
### Table 1 (Continued)

<table>
<thead>
<tr>
<th>Component</th>
<th>Composition</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum EssentialMedium Cat. # 410-1500</td>
<td>Earle's salts L-Glutamine Non-Essential Amino Amino Acids</td>
<td>1 pack of 96.1 gm</td>
</tr>
<tr>
<td>Deionized distilled water</td>
<td>4750 ml</td>
<td>Dissolve by gentle stirring at room temp.</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>22 gm (NaHCO₃)</td>
<td>2.2 gm/lt. with continuous stirring</td>
</tr>
<tr>
<td>Deionized distilled water</td>
<td>Add to make 5000 ml</td>
<td></td>
</tr>
<tr>
<td>pH adjustment</td>
<td>use 1N NaOH or 1N HCl</td>
<td>Adjust pH to 0.2-0.3 below desired level b</td>
</tr>
<tr>
<td>Sterilization</td>
<td>Filtration through methyl cellulose filter</td>
<td>Use 200 nm pore filter c</td>
</tr>
<tr>
<td>Result</td>
<td>Double strength MEM</td>
<td>Store at 4°C</td>
</tr>
<tr>
<td>To use</td>
<td>dilute 1:2 with deionized distilled water</td>
<td></td>
</tr>
</tbody>
</table>

b pH will rise 0.1-0.3 after filtration.

c Millipore Corporation, Bedford, Massachusetts.
Table 2. Antibiotics used in Minimum Essential Medium

<table>
<thead>
<tr>
<th>Pharmaceutical name</th>
<th>Trade name</th>
<th>Producer</th>
<th>Quantity used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin-G</td>
<td>Crystalline Penicillin G-potassium</td>
<td>E.R. Squibb &amp; Co. Inc., Princeton, NJ 08540</td>
<td>100 units/ml</td>
</tr>
<tr>
<td>Gentamicin Sulfate</td>
<td>Gentocin&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Schering Corp., Kenilworth, NJ 07033</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>Fungizone&lt;sup&gt;R&lt;/sup&gt;</td>
<td>E. R. Squibb &amp; Co, Inc., Princeton, NJ 08540</td>
<td>3.3 µg/ml</td>
</tr>
<tr>
<td>Streptomycin Sulfate</td>
<td>Streptomycin</td>
<td>Pfizer Inc. New York, NY 10017</td>
<td>100 µg/ml</td>
</tr>
</tbody>
</table>
Table 3. Preparation of Saline G

<table>
<thead>
<tr>
<th>Components</th>
<th>Quantity</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextrose</td>
<td>1.1 grams</td>
<td></td>
</tr>
<tr>
<td>Sodium chloride (NaCl)</td>
<td>8.0 grams</td>
<td></td>
</tr>
<tr>
<td>Potassium chloride (KCl)</td>
<td>0.4 grams</td>
<td></td>
</tr>
<tr>
<td>Sodium phosphate (Na₂HPO₄·7H₂O)</td>
<td>0.29 grams</td>
<td></td>
</tr>
<tr>
<td>Potassium phosphate (KH₂PO₄)</td>
<td>0.15 grams</td>
<td></td>
</tr>
<tr>
<td>Calcium chloride (CaCl₂·2H₂O)</td>
<td>0.016 grams</td>
<td></td>
</tr>
<tr>
<td>Lactalbumin hydrolysate</td>
<td>1.0 grams</td>
<td></td>
</tr>
<tr>
<td>Phenol red</td>
<td>0.0012 grams</td>
<td></td>
</tr>
<tr>
<td>Triple distilled water (deionized)</td>
<td>add to 1 liter</td>
<td>Gentle stirring, dissolve at room temperature</td>
</tr>
</tbody>
</table>

Adjust pH level to 0.2-0.3 below desired level using 1N sodium hydroxide or 1N hydrochloric acid. pH will rise after filtration by 0.1-0.3.

Sterilize solution by filtration through a methyl cellulose filter of 200 nm pore diameter.

Store at 4°C
Table 4. Composition and preparation of Phosphate Buffered Saline Solution (PBS)

<table>
<thead>
<tr>
<th>Materials</th>
<th>Quantity</th>
<th>Comments and Methodology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium phosphate (Na₂HPO₄)</td>
<td>4.60 gm</td>
<td>(0.008 M)</td>
</tr>
<tr>
<td>Sodium thiophosphate (NaH₂PO₄·H₂O)</td>
<td>1.10 gm</td>
<td>(0.002 M)</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>34.0 gm</td>
<td></td>
</tr>
<tr>
<td>Deionized distilled water</td>
<td>4 liters</td>
<td></td>
</tr>
</tbody>
</table>

Dissolve by gentle continuous stirring at room temperature

Sterilize by autoclaving at 121°C for 20 minutes. For pH adjustment, use 1N sodium hydroxide or 1N hydrochloric acid to 7.0-7.3
Table 5. Composition and preparation of trypsin-verseine solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextrose</td>
<td>2.0 grams</td>
<td></td>
</tr>
<tr>
<td>Sodium chloride (NaCl)</td>
<td>16.0 grams</td>
<td></td>
</tr>
<tr>
<td>Potassium chloride (KCl)</td>
<td>0.8 grams</td>
<td></td>
</tr>
<tr>
<td>Sodium bicarbonate (NaHCO₃)</td>
<td>1.4 grams</td>
<td></td>
</tr>
<tr>
<td>Versene a</td>
<td>0.4 grams</td>
<td></td>
</tr>
<tr>
<td>Phenol red 1% solution</td>
<td>2.0 ml</td>
<td></td>
</tr>
<tr>
<td>Deionized triple distilled water</td>
<td>180.0 ml</td>
<td>Mix and dissolve with gentle stirring at room temperature. Then heat to 37°C before the trypsin addition.</td>
</tr>
<tr>
<td>Trypsin b 1:250</td>
<td>1 gram</td>
<td>From hog pancreas, porcine parvovirus free. Mix with continuous stirring until trypsin is dissolved completely, approximately 1 hour</td>
</tr>
</tbody>
</table>

Add deionized triple distilled water to make 200 ml

Sterilize by filtration through 200 nm pore diameter methyl cellulose filter c. Result is 10X the needed concentration of trypsin-verseine solution. To use, dilute 1:10 in sterile deionized triple distilled water

a Disodium ethylene diamine tetracetate, Nutritional Biochemical Corp., Cleveland, Ohio.

b Gibco, Grand Island Biological Company, Grand Island, New York.

c Millipore Corporation, Bedford, Massachusetts.
Table 6a. The fomites and their sources used in study

<table>
<thead>
<tr>
<th>Fomite</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steel</td>
<td>Stainless steel bucket</td>
</tr>
<tr>
<td>Concrete</td>
<td>Concrete flooring</td>
</tr>
<tr>
<td>Wood</td>
<td>Wood bar and swine bedding</td>
</tr>
<tr>
<td>Soiled bedding</td>
<td>Animal house-husbandry area</td>
</tr>
<tr>
<td>Loam soil</td>
<td>Ames, Iowa</td>
</tr>
<tr>
<td>Polypropylene plastic</td>
<td>Plastic milk jug</td>
</tr>
<tr>
<td>Vinyl rubber</td>
<td>Rubber boots</td>
</tr>
</tbody>
</table>

Table 6b. The diluents used in this study

<table>
<thead>
<tr>
<th>Diluents</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline-G</td>
<td>Laboratory prepared</td>
</tr>
<tr>
<td>Physiological Saline Solution (PSS)</td>
<td>Laboratory prepared</td>
</tr>
<tr>
<td>Saliva</td>
<td>Pseudorabies virus free young pigs</td>
</tr>
<tr>
<td>Nasal washings</td>
<td>Pseudorabies virus free young pigs</td>
</tr>
<tr>
<td>Pharmaceutical name</td>
<td>Trade name</td>
</tr>
<tr>
<td>----------------------</td>
<td>------------</td>
</tr>
</tbody>
</table>
| chlorhexidine        | Nolvasan$^R$ | 1,1-Hexamethylene bis[5(p-chlorophenyl)biguanide] diacetate - 2%  
|                      |            | Inert ingredients - 98% |
| Quaternary           | Roccal$^R$ | n-alkyldimethylbenzyl ammonium chlorides - 50%  
|                      |            | ($\text{C}_{12}$ 67%, $\text{C}_{14}$ 25%, $\text{C}_{16}$ 7%,  
|                      |            | $\text{C}_8$, $\text{C}_{10}$, $\text{C}_{18}$ 1%) |
| Phenolic compound    | lstroke$^R$ | O-phenylphenol - 10%  
|                      | Environ$^R$ | O-benzyl-p-chlorophenol - 8.5%  
<p>|                      |            | p-tertiary-amylphenol - 2.0% |
| Alkali               | Lye        | Sodium hydroxide |</p>
<table>
<thead>
<tr>
<th>Chemical composition</th>
<th>Manufacturer</th>
<th>Recommended concentration (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FT. DODGE LABORATORIES, FORT DODGE, IOWA</td>
<td>3 oz/gallon</td>
<td>23437.5 ppm</td>
</tr>
<tr>
<td>STERLING Winthrop, 1/625 Inc., NY, NY</td>
<td>1600 ppm</td>
<td></td>
</tr>
<tr>
<td>VESTAL LABORATORIES, ST. LOUIS, MO 63110</td>
<td>3906.25 ppm</td>
<td></td>
</tr>
<tr>
<td>MANY</td>
<td>2%</td>
<td>21300 ppm</td>
</tr>
</tbody>
</table>
METHODS

Cell Culture Preparations

After confluent monolayers of MDBK cells were grown in 250 ml flasks (Corning Glass Works, Corning, New York), the supernatant GM was decanted and the cells removed with sufficient quantities of 1X trypsin-verse solution (7-10 ml). The cell-trypsin-verse solution were then transferred to 50 ml conical plastic centrifuge tubes, and centrifuged at 600 revolutions per minute (rpm) for 10 minutes. The cells settled down as pellets and the trypsin-verse solution was decanted and discarded. The cell pellets were resuspended again in centrifuge tubes in 20 ml fresh GM with gentle mixing so as not to harm the cells, then transferred to sufficient quantities of GM to make dilutions three times greater than hitherto.

From these last cell suspensions, the required number of single use eight-well plates (Lux Plates, Lux Scientific Corporation, Newbury Park, California), were inoculated at the rate of 2 ml per well, i.e., 16 ml per single plate. The next passage of the cells was commonly made by inoculating new 250 ml flasks with 20 ml of the same cell suspensions.

The plates and flasks were incubated in a humid chamber at 37°C with 5% carbon dioxide atmosphere with the screw caps kept loosened to maintain gaseous exchange. Confluent layers
of MDBK cells were usually grown up within 36-48 hours.

Fomite Preparations

All the fomites used, as shown in Table 6a, were filed or cut into small pieces of 1-2 mm$^2$ as in the case of steel, plastic or rubber, ground into fine particles as in the case of concrete or soil, or used in natural condition as in the case of soiled bedding which could easily be mixed into solution. These preparations helped make accurate measurements of the fomite materials possible through the experiments, made possible the placing of them in maximum contact with the virus-diluent-disinfectant combinations, made possible the keeping of the entire system at desired moisture-temperature-pH levels, and permitted sampling as desired.

Disinfectant Preparations

All the disinfectants were diluted with distilled water to 10X and 2X the RCFU, which were then diluted in the mixture with the virus-diluent-fomite mixtures and tested at 0.001, 0.01, 0.1 and 1X of the RCFU.
Diluents

Saliva and nasal washings

Saliva and nasal washings were stored at -20°C; then 24 hours before an experiment, the needed quantity was removed from -20°C to 4°C and then to 25°C or 4°C, the working temperatures in a water bath or ice bath, respectively.

Saline G, PSS, distilled water and PBS

These were kept at 4°C and warmed to 25°C in a water bath before the start of experiments, or maintained at 4°C in an ice bath.

Virus

The vials containing the virus aliquots were kept at -90°C. Just prior to use, the needed vials were thawed to 4° or 25°C to minimize virion damage and loss of titers.

Virus Titration

Virus dilutions

Sets of 6 numbered 126 x 16 millimeter test tubes were placed in racks in an ice bath, each containing 4.5 ml of saline G. Then 0.5 ml of freshly thawed stock virus was transferred to the first test tube with a sterile 1 ml pipette. Another sterile pipette was used to thoroughly mix the virus-saline G in the first test tube to ensure the even
distribution of the virions within the suspension. Then 0.5 ml was transferred to the second test tube. Further, serial 10-fold dilutions were made in the same manner, preparing $10^{-1} - 10^{-6}$ dilutions of the stock virus.

**Virus titration**

Titration of the virus was performed by inoculating 1.0 ml of each virus dilution into each of 2 cell culture wells, incubating 48 hours and counting the plaques. Experimental tests were performed using $10^{-2}$ final dilution of the stock virus throughout.

**Cell Culture Inoculation**

Confluent monolayers of MDBK cells between 36 and 48 hours after seeding in eight-well plastic plates were assigned randomly to each of the decimal virus dilutions, using 2 replicate wells per dilution. Two control wells were also used for each component of the tests; i.e., virus, fomites, diluents and growth and overlay media.

The GM covering the cells was aspirated and one ml from each dilution, starting with the highest dilution, was inoculated into each of the two test wells. The plates were then incubated for one hour adsorption time in a humid chamber at 37°C with 5% carbon dioxide atmosphere. After the adsorption, the inocula, diluents and GM were aspirated
from the inoculated wells and replaced with overlay media. The plates were reincubated in the humid chamber for another 48 hours for virus replication in the infected cells and formation of plaques.

**Fixation**

The plates were taken out of the humid chamber to room temperature and the cell layers were fixed with 2 ml per well of approximately 6% weight/volume of formalin solution for 20 minutes.

**Staining**

The media-formalin mixtures were discarded and the cell layers were stained with 1-2 ml of 2% crystal violet aqueous solution for ten minutes. Then the crystal violet was discarded and the plates were washed under tap water and air dried. The plaques in the countable wells were counted, averaged and multiplied by the virus dilution to calculate the titer of active pseudorabies in the inoculum.

**Virus Assay Techniques**

The assessment of disinfectant, fomite, diluent and temperature effects on the pseudorabies virus (PRV), as well as titrations and identification by serum naturalization tests was performed by plaque assay technique for active virus in the cell cultures (MDBK cells). These plaques
were formed as the virus was replicated in the infected cells, causing a cpe with cell destruction and formation of diagnostic plaques of 1.5-2 mm diameter.

The Effects of Test Diluents

Four sets of six, 126 x 16 millimeter test tubes were placed in racks in an ice or water bath at either 4°C or 25°C, respectively. Each set was assigned randomly to one of the test diluents listed in Table 6b. From each diluent, 4.5 ml were placed into first test tube of the assigned set. Then, 0.5 ml of the PRV suspension was added to the first tube of each dilution. Progressive 0.5 ml transfers produced serial 10-fold dilutions of the virus in each diluent. The serial dilutions were then assayed on MDBK cell layers as described for the virus assay technique. The pH was monitored using a Chem-Mat (Beckman Instruments, Inc., Fullerton, CA 92634), pH meter.

The Effect of the Combined Fomites and Diluents

The combined effects of the fomites and diluents against PRV were investigated in these experiments to assess their actions.

Samples of 0.5 gm each of the test fomites listed in Table 6a were added to 4.0 ml of each of the four test
diluents listed in Table 6b in 126 x 16 millimeter sterile glass test tubes. Then, the test tubes were placed in racks in either an ice bath (4°C) or water bath (25°C). A vial of the stock virus was thawed and diluted 1:10 in saline G, making the virus working solution. Then 0.5 ml of this virus working solution was added to each fomite-diluent test tube, resulting in a ratio of 1:1:8 virus-fomite-diluent, respectively. The mixtures were agitated thoroughly using hand shaking, mechanical shaking or Vortex (Scientific Products, American Hospital Supply Corporation, McGow Park, Ill. 60085), shaking for one minute.

After three minutes reaction time, the test tubes containing the reacted mixtures were centrifuged for 2 minutes at 1000 rpm. The solid materials were sedimented and 0.5 ml from the clear supernatant solutions was transferred to 4.5 ml PSS diluent at the same temperature, and after thorough mixing 0.5 ml was transferred from this to another test tube, resulting in two decimal dilutions of the reacted mixtures.

From each test tube, i.e., undiluted supernatant, 1:10 and 1:100 dilutions, 1.0 ml samples were inoculated on random bases per well on the monolayer of MDBK cells to assay for remaining active virus as described earlier. Wells with not over 600 countable plaques at the lowest inoculated
The Effects of the Test Disinfectants

Effect of the test disinfectants on the cell culture

Stock solutions of each disinfectant were made at 10 times the RCFU in distilled water (Table 7). Working dilutions were made at 1X and 0.1, 0.01 and 0.001 of the RCFU in PSS. Samples of 1.0 ml of each dilution were inoculated on MDBK cell monolayers. After one hour adsorption time, the inocula were aspirated and replaced with 2 ml per well of overlay medium and the plates were incubated in a humid chamber at 37°C with 5% carbon dioxide atmosphere. After 48 hours, the cell layers were fixed, stained and examined for levels of cell injury by the test disinfectants.

Effects of the combined disinfectants, fomites and diluents on pseudorabies virus

Preparation of disinfectant solutions

The stock 10X or 2X dilutions based on the RCFU of the disinfectants were diluted to final dilutions of 1X, 0.1, 0.01 and 0.001 of the RCFU in test mixtures with virus; fomites and diluents.
Preparation of the virus test suspensions

For each experiment, one vial of stock PRV was thawed and diluted to 10⁻¹ in saline G. This was diluted to 10⁻² final dilution in the test mixtures with disinfectants, fomites and diluents.

Preparation of the fomite test suspensions

Powdered, ground, cut or unprocessed fomites were mixed in 0.5 gm samples with 4.5 ml of test virus-disinfectant-diluent mixtures to give a final 10⁻¹ w/v suspension.

Preparation of the diluent suspensions

Diluents were placed in 3.5 ml aliquot in the test tubes for each experiment. The addition of virus, disinfectants and fomites gave final test suspensions of 1:1:1:7 of virus, disinfectants, fomites and diluents, respectively.

Reaction of the test combinations

The order of placing the test components was initially the diluents, then the fomites and then either virus followed by disinfectants or in most experiments, disinfectants followed by virus. Pipet mixing was used, followed by 3 or 28 minutes reaction time and 2 minutes further reaction time during centrifugation.
Preparation of the post-reaction dilutions

Immediately following centrifugation, 1:10 and 1:100 dilutions were made in physiological saline solution, always transferring 0.5 ml into 4.5 ml. Cell cultures were inoculated, placing 1.0 ml from each of the original reacted mixtures and the 1:10 and 1:100 dilutions on each of 2 wells of the cell culture plates. Following 1 hour adsorption time in a humid chamber at 37°C with 5% carbon dioxide atmosphere, the inocula were removed and replaced with overlay medium. The cell monolayers were then incubated under the same conditions for 48 hours, fixed and stained as discussed above. Plates to be read were selected on the bases of the lowest post-reaction dilutions; i.e., original reacted mixture, 1:10 or 1:100 which was not cytotoxic and in which the highest number of plaques was below 600 per well. In most tests, this was the 1:100 dilution but in some it was the 1:10 or the original reacted mixture.
RESULTS

Effects of Environmental Factors on PRV Survival

PRV survival outside the living host, as studied in 1979 by Davies (32), was found to be temperature and pH dependent (Figure 1). The virus was most stable at pH 6.2-7.7, and even at room temperature stayed at an infectious level more than 22 days. However, at extreme levels of pH 4.3 or 9.7, the virus was highly unstable. At 4°C, the virus was stable at levels of pH 6.2-8.8 for more than 2 months before it dropped below the infectious level.

Fomites common to hog raising operations, as well as liquids, including liquid wastes that might be contaminated with PRV, were found to affect the virus survival (Table 8), and might give support or inhibition for an extended period of time.

Pseudorabies virus (PRV) was suspended in PSS diluent at 25°C and mixed by pipet, hand or VortexR shaking for 5 minutes with the fomites used in this study (Figure 2). The virus survival was found to be greatly affected by pH levels and intensity of contact. With the first two methods of shaking, there were no significant differences in virus titer between solutions without fomites (control) and those with fomites other than concrete. In the presence of PSS,
Figure 1. Effects of environmental factors (pH and temperature) on PRV survival, using saline G diluent at 4° and 25°C and pipet mixing
Fig. (1-a)

25°C  77°F

Days

Virus Survival Log10

Fig. (1-a)

Fig. (1-b)

4°C  39°F

Days

Virus Survival Log10

Fig. (1-b)
Table 8. Survival of PRV in contact with selected fomites in three suspending fluids, 25°C, pipet mixing, minimum infective titer $10^{-1} \text{TCID}_{50}/\text{ml}$ (Freund and Beran, 1980)

<table>
<thead>
<tr>
<th>Fomite</th>
<th>Survival at infectious level in days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In saline G</td>
</tr>
<tr>
<td>Concrete</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Rubber</td>
<td>7</td>
</tr>
<tr>
<td>Sawdust bedding</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Plastic</td>
<td>8</td>
</tr>
<tr>
<td>Straw bedding</td>
<td>3</td>
</tr>
<tr>
<td>Loam soil</td>
<td>4</td>
</tr>
<tr>
<td>Steel</td>
<td>18</td>
</tr>
<tr>
<td>Denim cloth</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Green grass</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Shelled corn</td>
<td>36</td>
</tr>
<tr>
<td>Pelleted hog feed</td>
<td>3</td>
</tr>
<tr>
<td>Meat and bone meal</td>
<td>5</td>
</tr>
<tr>
<td>Alfalfa hay</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Feces</td>
<td>2</td>
</tr>
</tbody>
</table>
Figure 2. Effects of contact intensity with each of the fomites tested on PRV survival, using PSS diluent at 25°C for 5 minute virus contact
Fig. (2)
concrete had generated a high alkaline pH (11.6-12.2) which inactivated the virus within 5 minutes with any method of mixing. With 10X concentration of PBS solution, in contact with the concrete, the pH was brought down to 8.0 ± 0.2 resulting in no detectable depression in virus titer within the time of the test as compared to the control solution by pipet mixing; and only ≈0.5 log by hand shaking. Hydrochloric acid was used to lower the pH of PSS in contact with concrete to pH 7.4, but pipet, hand and vortex shaking raised it again to 8.4, 8.9 and 9.4, respectively, resulting in significant depression of the virus titer. Vortex shaking decreased the PRV titer with some fomites, by lowering the pH of PSS in contact with new wood to 4.9 and by increasing contact of virus with loam soil particles.

Fomite-diluent effects on PRV survival within one hour contact as compared with no fomite (Figure 3), were studied at 25°C, using pipet mixing. As PSS contains no buffer activity, the virus was subjected to the direct effects of the fomites, resulting in instant inactivation with concrete, sharp decline in virus titer with new wood, and significant variability in titer with some other fomites. Saline G which has minimal buffer activity, provided some virus protection; it did not compensate for the alkalinity caused by concrete; it decreased the new wood effect, and the
Figure 3. Effect of contact time on PRV suspended in each of the four diluents tested with no fomite and six fomites at 25°C using pipet mixing
variability from contact with other fomites. Saliva without fomite contact showed some inactivating activity, but was found to be the most protective diluent for the virus from the effects of concrete and new wood as well as other fomites. The virus suspended in saliva in contact with new wood and loam soil survived better than in saliva alone. Nasal washing also showed inactivating activity to PRV in the absence of any fomite, and as with PSS, provided no protection for the virus from the action of concrete, resulting in instant inactivation of the virus. In contact with new wood or other fomites, the virus in nasal washings showed significant variability in its survival. However, in the presence of soiled bedding, the virus survived better than in nasal washing alone.

The high level of alkaline pH (11.8 ± 0.4) caused by concrete fomite was found to be difficult to change. Repeated washings of weathered concrete with PSS diluent and vortex shaking for various lengths of time intervals (Figure 4) dropped the pH slightly when the PSS was repeatedly changed. However, as the period of contact between washings was extended, the pH was increased and maintained at high level for a long time. Thus, as seen from Figures 2, 3, and 4, clean concrete strongly inactivated PRV by the high alkaline pH exerted by this fomite.
Figure 4. Effect of washing of concrete on pH of washing fluids. PSS washing fluid, 25°C, Vortex shaking arrows (↑) indicate times at which washing fluid was removed and replenished with fresh fluid.
Fig. (4)
Disinfectant Activity Against PRV

The working dilutions of the four disinfectants used through this research work were based on experimental results (Figure 5). A fixed quantity of PRV suspended in PSS titrated against two-fold dilutions of the four disinfectants starting at the RCFU, at 25°C with 5 minutes contact without any fomite was effective through the following dilutions:

- Roccal to 1:128 from RCFU
- Nolvasan to 1:256 from RCFU
- I Stroke Environ to 1:256 from RCFU
- Sodium hydroxide to 1:2048 from RCFU

The 10-fold dilutions required for this study calculated in ppm are presented in Table 9.

Application of the test disinfectants pre- or post-disease appearance, had some positive or negative effects on their disinfecting activity, depending on the disinfectant type (Figure 6). Soiled bedding was chosen to show those effects at 25°C with PSS diluent for 5 minutes contact. Nolvasan and I Stroke Environ were found to be more effective if used before contamination of the fomite with virus. Roccal and sodium hydroxide were more effective when used after the soiled bedding was contaminated with virus.
Figure 5. Survival of PRV in contact with two-fold dilutions of disinfectants. PSS diluent, 25°C, 5 minutes contact, pipet mixing.
Table 9. Dilutions used to study the disinfectant activity against PRV, determined from their manufacturers' recommended concentrations for field use (RCFU)

<table>
<thead>
<tr>
<th>Dilution used</th>
<th>Disinfectants Concentration (in ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>'Nolvasan'</td>
</tr>
<tr>
<td>RCFU</td>
<td>23427.5</td>
</tr>
<tr>
<td>I. Two-fold</td>
<td></td>
</tr>
<tr>
<td>1/64</td>
<td>366.2</td>
</tr>
<tr>
<td>1/128</td>
<td>183.1</td>
</tr>
<tr>
<td>1/256</td>
<td>91.55</td>
</tr>
<tr>
<td>1/512</td>
<td>45.775</td>
</tr>
<tr>
<td>1/1024</td>
<td>22.88</td>
</tr>
<tr>
<td>1/2048</td>
<td>11.44</td>
</tr>
<tr>
<td>II. Ten-fold</td>
<td></td>
</tr>
<tr>
<td>1:10</td>
<td>2343.75</td>
</tr>
<tr>
<td>1:100</td>
<td>234.375</td>
</tr>
<tr>
<td>1:1000</td>
<td>23.4375</td>
</tr>
</tbody>
</table>
Figure 6. Effects of pre- and post-disinfection of PRV-contaminated-soiled bedding on each of the 4 disinfectants' activity using PSS diluent and pipet mixing at 25°C for 5 minute virus contact
DISINFECTANT CONCENTRATION

VIRUS SURVIVAL LOG_{10}

Fig. (6-a)

- Nolvasan
- Roccal
- I. Stroke Environ.
- Sod. Hydroxide

DISINFECTANT CONCENTRATION

VIRUS SURVIVAL LOG_{10}

Fig. (6-b)
Effects of Diluents on Disinfectant Activity Against PRV, with and without Fomites

Pseudorabies virus (PRV) was suspended in 4 diluents and subjected at 25°C to 4 disinfectants in the absence and presence of different fomites for 5 or 30 min. contact time, using pipet mixing (Figures 7-10).

The disinfectant activity against PRV suspended in 4 test diluents at 25°C was tested for 5 min. contact without any fomite (Figure 7). Nolvasan, Roccal and I Stroke Environ were effective at 1:10 dilutions of their RCFU against PRV suspended in PSS or saline G and 1:100 dilutions of their RCFU against virus suspended in nasal washings. Sodium hydroxide was effective at 1:10 dilution of its RCFU in the presence of each of the 4 diluents. Saliva appeared to be significantly more protective for the virus than the other test diluents against all 4 disinfectants tested.

Effects of PSS were determined in the presence of fomites on disinfectant activity (Figure 8). Nolvasan and Roccal were bound by loam soil and about 90% of their disinfecting activity was inhibited by new wood and soiled bedding as compared with no fomite control. However, in the absence of these disinfectants, new wood and to a lesser extent, loam soil were quite toxic to the virus alone in PSS. About 90% of I Stroke Environ activity was inhibited also by the fomites tested. Sodium hydroxide activity
was inhibited by new wood but improved by soiled bedding and rubber with presence of PSS diluent.

Effects of saline G were assessed in the presence of fomites (Figure 9). Nolvasan and Roccal activity were tied up by loam soil and their disinfecting activity reduced about 90% by new wood and soiled bedding in the presence of saline G as diluent. Although the virus titer declined rapidly in contact with new wood without disinfectants (Figure 3), I Stroke Environ activity was improved by new wood but inhibited by loam soil and soiled bedding. Sodium hydroxide retained its activity and no detectable virus at 1:10 dilution from RCFU appeared with or without fomites.

Effects of saliva were determined in the presence of fomites (Figure 10). Nolvasan and Roccal activities were bound by new wood and loam soil, and the virus retained the same titer as seen in the absence of these disinfectants (Figure 3). The activity of Nolvasan and Roccal was approximately 90% inhibited by soiled bedding. Disinfecting activity of Nolvasan and Roccal was however, increased by plastic grate fomite in the presence of saliva as diluent compared to no fomite. I Stroke Environ and sodium hydroxide activities also were bound or inhibited by new wood and improved by plastic grate in the presence of saliva as diluent.
Figure 7. Effects of each of the 4 disinfectants against PRV survival, suspended in each of the 4 diluents tested without fomite at 25°C for 30 minute contact, using pipet mixing.
Fig. (7-a) NOLVASAN

Fig. (7-b) ROCCAL

Fig. (7-c) I STROKÉ ENVIRON

Fig. (7-d) SODIUM HYDROXIDE
Figure 8. Effects of PSS diluent on each of the 4 disinfectants' activity against PRV in contact with each of the fomites tested at 25°C for 30 minute contact, using pipet mixing
--- No Fomite
- Plastic Grate
- Concrete
- Soil
- Soiled Bedding
- Rubber
- New Wood

**Fig. (8-a) NOLVASAN**

**Fig. (8-b) ROCCAL**

**Fig. (8-c) I STROKE ENVIRON.**

**Fig. (8-d) SODIUM HYDROXIDE**
Figure 9. Effects of saline G diluent on each of the 4 disinfectants' activity against PRV in contact with each of the fomites tested at 25°C, for 30 minute contact, using pipet mixing
--- No Fomite

Plastic Grate

Soil

Soiled Bedding

Rubber

New Wood

VIRUS SURVIVAL LOG_{10}

DISINFECTANT CONCENTRATION

Fig. (9-a) NOLVASAN

DISINFECTANT CONCENTRATION

Fig. (9-b) ROCCAL

DISINFECTANT CONCENTRATION

Fig. (9-c) I STROKE ENVIRON.

DISINFECTANT CONCENTRATION

Fig (9-d) SODIUM HYDROXIDE

0.001 0.01 0.1 1x

0 1 2 3 4 5 6 7

0 1 2 3 4 5 6 7
Figure 10. Effects of saliva diluent on each of the 4 disinfectants' activity against PRV in contact with each of the fomites tested at 25°C for 30 minute contact, using pipet mixing
--- No Fomite
--- Concrete
Plastic
Soil
Soiled Bedding
Rubber
New Wood

DISINFECTANT CONCENTRATION
Fig. (10-a) NOLVASAN

DISINFECTANT CONCENTRATION
Fig. (10-b) ROCCAL

DISINFECTANT CONCENTRATION
Fig. (10-c) I STROKE ENVIRON.

DISINFECTANT CONCENTRATION
Fig. (10-d) SODIUM HYDROXIDE
Effect of Fomites on Disinfectant Activity against PRV in the Four Tested Diluents

The effect of the test fomites on the four disinfectants against PRV was tested in the presence of four diluents at 25°C for 30 min., using pipet mixing (Figures 11-14).

New wood (Figure 11), in the presence of saliva as diluent bound all four disinfectants resulting in virus protection as if there were no disinfectants (Figure 3). In the presence of PSS, saline G and nasal washings, new wood resulted in a 10-100-fold decrease in activity of Nolvasan, Roccal and sodium hydroxide as compared to their action in the absence of any fomite (Figure 7). However, I Stroke Environ retained its full activity in the presence of diluents, other than saliva as if no fomites were present (Figure 7).

Plastic grate (Figure 12) generally had no significant effect on the action of the four disinfectants in the presence of PSS, saline G and nasal washing diluents from that of no fomite (Figure 7). In the presence of saliva, however, plastic grate significantly improved the activity of the four test disinfectants (Figure 7). In the absence of disinfectants, plastic grate in contact with saliva (Figure 3), showed no difference from saliva alone in viral stability.

Soiled bedding (Figure 13), in the presence of the four diluents, decreased the activity of Nolvasan, Roccal, and
I Stroke Environ 10-100-fold from their activities without fomite (Figure 7), and no detectable virus was found only with the use of the disinfectants at their RCFU. However, the activity of sodium hydroxide was increased 10-fold in contact with PSS from that without fomite (Figure 7); one-tenth of the RCFU of this disinfectant completely inactivated the virus in the presence of all four diluents.

Loam soil (Figure 14), in the presence of the four diluents, tied up Nolvasan and Roccal and decreased 10-fold the activity of I Stroke Environ from that without any fomite (Figure 7). Loam soil, in the absence of disinfectants (Figure 3), showed virus protection with saliva only, but in the presence of PSS, saline G and nasal washing, there was significant inactivation of the virus. Sodium hydroxide showed no difference in activity against PRV in contact with loam soil in the presence of the four diluents from that without any fomite (Figure 7).

Effect of Temperature with and without Fomites on Disinfectant Activity against PRV

The action of the four test disinfectants against PRV, with and without fomites, was tested at 4°C and 25°C in the presence of saline G as diluent for 30 min. contact with pipet mixing (Figures 15-19).
Figure 11. Effects of new wood fomite on each of the 4 disinfectants' activity against PRV suspended in each of the 4 diluents tested at 25°C for 30 minute contact, using pipet mixing.
Figure 12. Effects of plastic grate fomite on each of the 4 disinfectants' activity against PRV suspended in each of the 4 diluents tested at 25°C for 30 minute contact using pipet mixing
Fig. (12-a) NOLVASAN
Fig. (12-b) ROCAL
Fig (12-c) I STROKE ENVIRON.
Fig (12-d) SODIUM HYDROXIDE
Figure 13. Effects of soiled bedding fomite on each of the 4 disinfectants' activity against PRV suspended in each of the 4 diluents tested at 25°C for 30 minute contact using pipet mixing.
Fig. (13-a) NOLVASAN
Fig. (13-b) ROCCAL
Fig. (13-c) I STROKE ENVIRON.
Fig. (13-d) SODIUM HYDROXIDE
Figure 14. Effects of loam soil fomite on each of the 4 disinfectants' activity against PRV suspended in each of 4 diluents tested at 25°C for 30 minute contact using pipet mixing.
Fig. (14-a) Disinfectant concentration effects on virus survival.

- **PSS**
- **Saline G**
- **Nasal Washing**

Fig. (14-b) Disinfectant concentration effects on virus survival.

Fig. (14-c) Disinfectant concentration effects on virus survival in stroke environment.

Fig. (14-d) Disinfectant concentration effects on virus survival with sodium hydroxide.

DISINFECTANT CONCENTRATION

VIRUS SURVIVAL LOG10

0 0.001 0.01 0.1 1x

DISINFECTANT CONCENTRATION

VIRUS SURVIVAL LOG10

0 0.001 0.01 0.1 1x

DISINFECTANT CONCENTRATION

VIRUS SURVIVAL LOG10

0 0.001 0.01 0.1 1x

DISINFECTANT CONCENTRATION

VIRUS SURVIVAL LOG10

0 0.001 0.01 0.1 1x

DISINFECTANT CONCENTRATION

VIRUS SURVIVAL LOG10

0 0.001 0.01 0.1 1x

DISINFECTANT CONCENTRATION

VIRUS SURVIVAL LOG10

0 0.001 0.01 0.1 1x

DISINFECTANT CONCENTRATION

VIRUS SURVIVAL LOG10

0 0.001 0.01 0.1 1x

DISINFECTANT CONCENTRATION

VIRUS SURVIVAL LOG10

0 0.001 0.01 0.1 1x

DISINFECTANT CONCENTRATION

VIRUS SURVIVAL LOG10

0 0.001 0.01 0.1 1x

DISINFECTANT CONCENTRATION

VIRUS SURVIVAL LOG10

0 0.001 0.01 0.1 1x

DISINFECTANT CONCENTRATION

VIRUS SURVIVAL LOG10

0 0.001 0.01 0.1 1x

DISINFECTANT CONCENTRATION

VIRUS SURVIVAL LOG10

0 0.001 0.01 0.1 1x

DISINFECTANT CONCENTRATION

VIRUS SURVIVAL LOG10

0 0.001 0.01 0.1 1x

DISINFECTANT CONCENTRATION

VIRUS SURVIVAL LOG10

0 0.001 0.01 0.1 1x

DISINFECTANT CONCENTRATION

VIRUS SURVIVAL LOG10

0 0.001 0.01 0.1 1x

DISINFECTANT CONCENTRATION

VIRUS SURVIVAL LOG10

0 0.001 0.01 0.1 1x
Without any fomite (Figure 15), Nolvasan was more effective at 25°C, but there was no significant temperature effect on Roccal, I Stroke Environ or sodium hydroxide at 4°C or 25°C. No detectable virus was recovered at either temperature from the four disinfectants at 1:10 dilution of their RCFU.

With new wood (Figure 16), Nolvasan and Roccal showed about 90% lowering of their disinfectant activity from that without any fomite but no differences were recorded at either temperature and no detectable virus remained at RCFU. I Stroke Environ was 10-fold more active at 25°C, with no detectable virus at 1:100 dilution (39 ppm) while 1:10 dilution (390.6 ppm) was needed to render the virus undetectable at 4°C. Sodium hydroxide was slightly more effective in the absence of any fomite (Figure 15) at 1:100 dilution, but no detectable virus was recovered at either temperature at 1:10 dilution of its RCFU.

With plastic grate (Figure 17), Nolvasan and Roccal showed better activity at 25°C. I Stroke Environ and sodium hydroxide showed no difference at either temperature. The four disinfectants were as effective in the presence as in the absence of any fomite (Figure 15) and no detectable virus was found at 1:10 dilution of their RCFUs.

With soiled bedding (Figure 18), Nolvasan showed slightly
better activity in the higher dilutions at 25°C, but in lower dilutions, no significant difference was recorded between its activity at the two temperatures. Roccal showed no significant difference in activity at either temperature and no detectable virus remained at the RCFU. I Stroke Environ also showed rapid inactivation of PRV in higher dilution at 25°C. At 4°C, no detectable virus remained at 1:10 dilution (390.6 ppm), while at RCFU (3906.5 ppm) at 25°C, the virus was undetectable. Sodium hydroxide showed no significant difference in activity at either temperature and no detectable virus remained at 1:10 RCFU at either temperature.

With loam soil (Figure 19), Nolvasan and Roccal showed more activity in higher dilutions at 4°C, and no significant difference at lower disinfectant dilutions between the two temperatures. I Stroke Environ was 10-fold more effective at 4°C than at 25°C, with no detectable virus at the RCFU at 25°C or at 1:10 dilution at 4°C. Sodium hydroxide was more effective in higher dilutions at 4°C than at 25°C but in lower disinfectant dilutions, no detectable virus appeared at either temperature.
Figure 15. Effects of temperature on each of the 4 disinfectants' activity against PRV suspended in saline G diluent without fomite at 4° and 25°C for 5 minute contact using pipet mixing.
DISINFECTANT CONCENTRATION
Fig. (15-a) NOLVASAN

DISINFECTANT CONCENTRATION
Fig. (15-b) ROCCAL

DISINFECTANT CONCENTRATION
Fig. (15-c) I STROKE ENVIRON.

DISINFECTANT CONCENTRATION
Fig. (15-d) SODIUM HYDROXIDE
Figure 16. Effects of temperature on each of the 4 disinfectants' activity against PRV suspended in saline in contact with new wood at 4° and 25°C for 30 minutes using pipet mixing.
-25°C  

---

4°C  

---

DISINFECTANT CONCENTRATION

Fig. (16-a) NOLVASAN

Fig. (16-b) ROCCAL

Fig. (16-c) 1 STROKE ENVIRON.

Fig. (16-d) SODIUM HYDROXIDE
Figure 17. Effects of temperature on each of the 4 disinfectants' activity against PRV suspended in saline G and in contact with plastic grate at 4° and 25°C for 30 minutes using pipet mixing.
Figure (17-a) NOLVASAN

Figure (17-b) ROCCAL

Figure (17-c) I STROKE ENVIRON.

Figure (17-d) SODIUM HYDROXIDE
Figure 18. Effects of temperature on each of the 4 disinfectants' activity on PRV suspended in saline G and in contact with soiled bedding at 4° and 25°C for 30 minutes using pipet mixing.
DISINFECTANT CONCENTRATION

Fig. (18-a) NOLVASAN

DISINFECTANT CONCENTRATION

Fig. (18-b) ROCCAL

DISINFECTANT CONCENTRATION

Fig (18-c) I STROKE ENVIRON.

DISINFECTANT CONCENTRATION

Fig. (18-d) SODIUM HYDROXIDE
Figure 19. Effects of temperature on each of the 4 disinfectants' activity against PRV suspended in saline G and in contact with loam soil at 4°C and 25°C for 30 minutes using pipet mixing.
Fig. (19-a) NOVASCAN
Fig. (19-b) ROCCAL
Fig. (19-c) I STROKE ENVIRON.
Fig. (19-d) SODIUM HYDROXIDE
Pseudorabies virus (PRV) survival outside the living host as well as some environmental factors that might affect its survival, have been discussed in previous studies (32, 44). The direct activities of many types of disinfectants against the virus have been well-documented and are discussed in the literature review. It has also been well-documented that environmental transmission of PRV to pigs occurs, and that pigs have been infected after being housed in animal barns which previously housed pigs with PRV infections. This research work was designed to study the activity of disinfectants in the presence of environmental factors, and to assess the interactions of disinfectants and environmental fomites in inhibiting the viral inactivating actions of both the fomites and the disinfectants, permitting PRV to survive longer at infectious levels.

The disinfectants used in this study represent four main categories of chemical compounds that have been mostly used for farm disinfection, namely chlorhexidine, quaternary ammonium compounds, phenol derivatives and alkalies. Those disinfectants have been found to be effective against PRV by many research workers as discussed in the literature review part of this writing.

As an enveloped virus in which the envelope is assembled
from the nuclear membrane, PRV is sensitive to a wide range of disinfectants and can be inactivated easily by disinfectants or agents with lipid dissolving capability, like Nolvasan and I Stroke Environ, cationic compounds like Roccal, alkalies like sodium hydroxide, as well as by anionic detergents and acids. Disinfectants, other than lipid solvents, also may act by disrupting the structure and ionization of the amine and carboxyl radicals of the virus envelope, resulting in destruction of virus attachment sites, and thus, inhibiting viral infectivity. However, in actual use, the activities of disinfectants may be inhibited by environmental components which might be found in the surrounding vicinity or where the virus is present, such as serum, blood, tissues, body fluids or other fomites that might inhibit disinfectants' actions.

Four diluents were used in this study. PSS and saline G, in which the virus was most stable at neutral pH, and at temperature near 25°C, while porcine saliva and nasal washing showed toxic inhibitory effects against the virus. These findings have been previously reported (44).

In the absence of fomites, the four disinfectants used in this study, Nolvasan, Roccal, I Stroke Environ and sodium hydroxide were found to be active against PRV; >10^6 TCID<sub>50</sub> of the virus suspended in each of the four diluents were
fully inactivated within 30 min. at 25°C by 1:10 to 1:100 dilutions of the RCFU of those disinfectants in all of the test diluents. However, porcine saliva and saline G, which either by their protein content, or buffer activity, respectively, protected the virus from low disinfectant concentrations; but porcine nasal washings with their toxicity added to the disinfectant activity, resulting in rapid virus inactivation at low disinfectant concentration. Only sodium hydroxide showed similar activity irrespective of the suspending fluids, inactivating the virus at 1:10 dilution of its recommended concentration in all test diluents.

PRV suspensions at pH levels mentioned (34) as favorable for PRV survival remained stable in the presence of plastic grate, loam soil and soiled bedding; concrete raised the pH; and new wood lowered the pH to levels at which virus was inactivated. Among these fomites, concrete and plastic grate enhanced the action of disinfectants tested; the other fomites inhibited disinfecting activity.

Clean concrete in the presence of PSS, saline G and nasal washing diluents generated a pH (11.8 ± 0.4) that resulted in rapid virus decay; disinfectant activity was greatly enhanced, instantly inactivating the virus. Saliva, which showed some inhibiting or deteriorating activity on PRV by itself, somewhat protected the virus from
concrete activity, but the concrete-disinfectant combined activity overcame the protection of saliva, leading to inactivation of the virus instantly also.

The PRV inactivating action of concrete was retained even with continuous washing with PSS and hydrochloric acid neutralization. But 10X concentration of PBS, a concentrated buffer solution with pH 7.0, controlled concrete activity and brought the pH down to 8.0 ± 0.2, where the virus survived without significant difference from the no fomite control within the time of the experiment. However, vigorous shaking generated additional concrete activity (pH 9.1) that resulted in >2 logs loss from the virus titer. Thus, the alkaline pH of concrete was considered to be the main contributing factor to virus decay, both by itself and in the presence of the test disinfectants as well. By and large, clean concrete retained its activity and capability of PRV inhibition for a long period of time that enhanced disinfectant activity.

Plastic grate fomite was previously shown (44) to inhibit PRV, especially in the presence of saliva. In the presence of plastic grate, disinfecting activity was enhanced, especially in the presence of saliva diluent, where the virus inactivation was uniquely rapid. The regular inhibiting activity of saliva, which was increased in the
presence of plastic grate, greatly increased the activity of all four disinfectants against PRV. The exact mechanism was not clearly understood; it was considered that there might be increased saliva enzymatic activity in the presence of plastic that resulted in increased virus vulnerability to disinfectant activity. Some increase in disinfectant activity against PRV was also shown by plastic grate fomite in the presence of nasal washing, saline G and PSS. Thus, plastic grate could be disinfected readily with any of the four test disinfectants without any problems.

Soiled bedding, due to the presence of organic material, decreased the activity of Nolvasan and Roccal against PRV by more than 90% in the presence of all four diluents; with only the test concentrations at their RCFU being effective in inactivating the virus. Thus, the use of these two disinfectants to disinfect soiled bedding would require concentrations at least at their RCFU levels; otherwise, they would not be effective. A 1:10 dilution of I Stroke Environ disinfectant resulted in no detectable virus in the presence of PSS, nasal washings and saliva diluents with soiled bedding, but, in the presence of saline G, this disinfectant lost about 90% of its activity and had to be applied at its RCFU for disinfecting soiled bedding. Sodium hydroxide retained its activity in contact with soiled bedding in the presence
of all four test diluents. Thus, sodium hydroxide and I Stroke Environ were effective for disinfecting soiled bedding if maintained in a good disinfecting concentration levels.

New wood, in the presence of PSS, saline G and nasal washings, decreased the activity of Nolvasan and Roccal against PRV by more than 90%, an effect also noted with sodium hydroxide in the presence of PSS. Only at their RCFUs were these disinfectants able to inactivate the virus. However, no significant change in I Stroke Environ activity against PRV was recorded by new wood in the presence of PSS, saline G or nasal washing diluents. In the presence of saliva, new wood bound the activities of all four disinfectants, maintaining the virus at an infectious level. Even at the RCFU strength of these disinfectants, the virus titer (initially >6.76 logs) was decreased only 1, 0.5, 0.4 and 0.25 logs, by the activities of sodium hydroxide, I Stroke Environ, Roccal and Nolvasan, respectively. This protective action of new wood was not present in the absence of disinfectants where new wood caused a rapid decline in the virus titer by itself in the presence of PSS, saline G and nasal washings, and a small decline by itself in the presence of saliva. This showed that wooden walls, bars, doors or bedding from wood shavings which have viral inactivating action in themselves might actually protect the
virus in the presence of saliva from the disinfectant activity of all four test compounds.

Loam soil tied up Nolvasan and Roccal at all dilutions tested, even at RCFU, protecting the virus from disinfectant action, especially in saliva and PSS, although the soil was in itself toxic to the virus. In loam soil, I Stroke Environ inhibited the virus at 1:10 dilution of its RCFU in the presence of PSS and saliva, but only at RCFU did it inhibit the virus in the presence of nasal washing and saline G. Sodium hydroxide retained its activity and inhibited the virus at 1:10 dilution of its RCFU in all diluents. Thus, Nolvasan and Roccal were not effective for disinfecting loam soil; I Stroke Environ might be helpful if maintained in high concentration and not diluted in soil to below the effective concentration. However, sodium hydroxide stood as the most effective disinfectant for loam soil against PRV.

The effects of temperature on disinfectant activity were tested at 4° and 25°C in the presence of saline G with and without fomites. Without fomites, Nolvasan showed more activity at 25°C than at 4°C. Roccal, I Stroke Environ and sodium hydroxide showed no difference in their activity at either temperature. With new wood, only I Stroke Environ was more effective at 25°C than at 4°C, other disinfectants showed no difference in their activities against PRV between
the two temperatures tested. With plastic grate, Nolvasan and Roccal were more active at 25°C, I Stroke Environ and sodium hydroxide showed no difference. Soiled bedding showed I Stroke Environ to be more effective at 25°C, and no difference in the activity of the other disinfectants at either temperature. With loam soil, all four disinfectants were more effective at 4°C, especially I Stroke Environ. Thus, fomites other than loam soil were preferably disinfected at 25°C with the disinfectant of choice for that fomite. Loam soil was most effectively disinfected at 4°C, with I Stroke Environ and sodium hydroxide being the most active of the test disinfectants on it.
CONCLUSIONS

Findings in this study have application in disease control of importance for the livestock industry. This work showed that the mere use of chemical disinfectants in farm environments does not mean that infective agents were destroyed or transmission of disease controlled. Depending on the environmental conditions, specific disinfectants may effectively inactivate infectious agents, may have little or no effectivity, or may even help in the protection of such agents in the environment. Clean concrete was effective in inactivating pseudorabies virus by itself, and with the use of low concentrations of any of the four disinfectants tested here, quickly inactivated PRV to below the detectable level. Thus, concrete should be kept clean and this will maintain its activity for a long time, acting together with the test disinfectants. Plastic grate as well showed no problem of disinfection, and could be easily disinfected with any of the test disinfectants. Soiled bedding reduced the antiviral activity of Nolvasan and Roccal and to a lesser extent of I Stroke Environ and sodium hydroxide. High levels of disinfectant were required to inactivate PRV in the presence of soiled bedding. New wood if kept clean, was capable of inactivating PRV in the absence of any protecting fluid by itself. Saliva, however, which is the
main natural carrier for PRV from shedding swine, was protective to the virus in contact with new wood and also protected it in a high infectious level from the action of the four disinfectants tested. Although I Stroke Environ was not inhibited by new wood in the presence of PSS, saline G and porcine nasal washing, the other test disinfectants lost more than 90% of their activities when absorbed by the wood fomite. New wood, or wood in general should be scrubbed very clean to promote its natural inhibition of PRV and then disinfected with chemical disinfectants in high concentrations, as they quickly lose their effectivity on wood below their RCFU. I Stroke Environ and sodium hydroxide were the most effective against PRV in saliva on new wood among the test disinfectants, especially at 25°C. With loam soil, Nolvasan and Roccal failed to show any significant effect against PRV in the presence of any of the four test diluents at either test temperature and thus, could not be recommended for this job. I Stroke Environ and sodium hydroxide were effective, if maintained at their RCFU, as soil disinfectants against PRV, especially at 4°C where they were more effective than at 25°C.

Generally, surfaces contaminated with PRV which are to be disinfected should be scrubbed, cleaned, washed and dried, before chemical disinfection to maintain the activity of
these disinfectants at their RCFU. Any of the four disinfectants was effective in disinfecting clean concrete and plastic. I Stroke Environ and sodium hydroxide were preferable for new wood and soiled bedding and were the only disinfectants showing effectiveness for loam soil disinfection.

Though a swine raising industry is not of economic importance in Iraq, the herpes viruses may become important in the very large poultry, cattle, sheep and goat industries. Much of the information gained in this study may have direct application to the control of diseases caused by herpes and other lipid containing viruses as well as those caused by other infectious agents in Iraq. The techniques developed for and used in this study may have application for local studies in my country.
LITERATURE CITED


ACKNOWLEDGMENTS

This study would not have been possible without the guidance and advice given by my major professor, Dr. G. W. Beran, who rather adventurously agreed to be my major professor without having met me and who has since given unestimable support throughout this project. I wish to thank him with deep appreciation. I wish to thank Dr. R. A. Packer who again without having met me accepted me into this department and served on my committee, and Dr. L. Christian, of the Animal Science Department for serving on my committee.

I wish to thank with appreciation, the loyalty and courage of my family, especially my wife Ahlam and sons Ahmed and Haider to whom I dedicate this work. They will understand that I cannot adequately express the warmth of my feelings for them in cold print.

I wish to thank Mrs. Dorothy Murphy for her efficient technical assistance and Pat Gunnells, whose efforts in typing and editing my thesis will always be appreciated.

I would lastly like to thank all of the personnel throughout the scientific and administrative community of this university who have helped me in my study.