

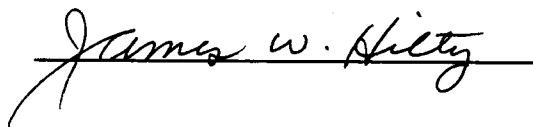
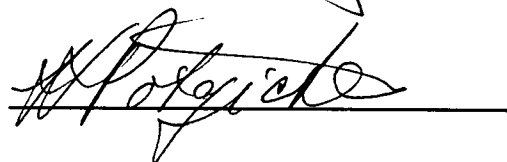
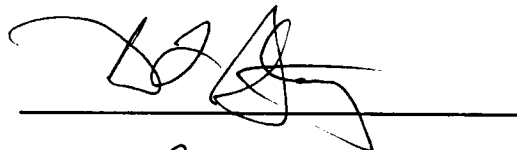
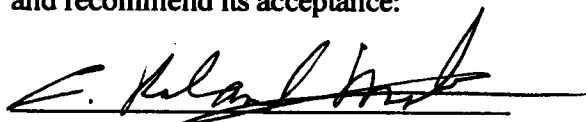
To the Graduate Council:

I am submitting herewith a dissertation written by Elizabeth J. Deming entitled "Use of an Animal Model in the Assessment of the Risk of Human Viral Disease Transmission Associated with Surface Application of Treated Wastewater onto Lawns". I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Ecology.



Dr. Robert von Bernuth, Major Professor

We have read this dissertation
and recommend its acceptance:



Accepted for the Council:



Vice Provost
and Dean of The Graduate School

**USE OF AN ANIMAL MODEL IN THE ASSESSMENT OF
THE RISK OF HUMAN VIRAL DISEASE TRANSMISSION
ASSOCIATED WITH SURFACE APPLICATION OF
TREATED WASTEWATER ONTO LAWNS**

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Elizabeth J. Deming

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DEDICATION

To my parents

Howard and Dorothy Deming

and

In memory of

Dr. Robert C. Pendleton

ABSTRACT

Land application of treated wastewater is increasing in the United States and developing countries. One of the major barriers to use of treated wastewater for irrigation is the uncertainty associated with potential disease transmission from contact with human enteric viruses in wastewater. This study evaluated the feasibility of using pigs as an animal model to assess the risk of human viral infection from land application of treated wastewater. The model system consisted of 3-week-old pigs and porcine enterovirus-serotype 2 (PE2). Pigs were used because the porcine digestive tract and the associated porcine enteroviruses are similar to those of man.

Oral challenge inoculation experiments were carried out to examine antibody production in pigs as a result of infection with PE2 virus. Piglets free of antibody were intragastrically inoculated with 0, 100 or 100,000 CCID₅₀ of PE2 virus. Serum was monitored to detect antibody to PE2 at 3, 7, 10 and 13 days post administration. By 13 days post administration all pigs challenge inoculated with as little as 100 CCID₅₀ of PE2 virus had seroconverted.

To evaluate the potential for using pigs to assess the risk of viral infection from contact with virus on lawns, piglets free of antibody to PE2 were placed on grass plots seeded with known concentrations of PE2 virus and serologically monitored for production of PE2 antibody. A total of 52 pigs were exposed for two hours to concentrations of 4×10^2 to 4×10^5 CCID₅₀ virus seeded to 0.36 m² grass plots. Incidence rates of infection were calculated for each exposure concentration in order to examine the nature of the dose-response relationship resulting from a single exposure to virus seeded to grass. The incidence rate of infection among pigs exposed for two hours to 4×10^5 CCID₅₀ PE2 virus seeded on grass was 66.7. The incidence rate

dropped off dramatically as the virus concentration seeded onto plots decreased. No pigs exposed to grass plots seeded with 400 CCID₅₀ of PE2 virus became infected.

The results demonstrated that pigs provide a simple, efficient means for providing an indication of the risk of viral infection to humans who may come in contact with virus in wastewater applied to lawns. Information gained from studies using pigs for this purpose can be used to examine the dose-response relationship and establish best practicable management procedures which will limit potential health and environmental effects from use of treated wastewater for irrigation.

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CHAPTER 1

INTRODUCTION

BACKGROUND

The United States, along with many countries throughout the world, is facing current or near-term shortages of freshwater supplies. Surface water and groundwater supplies are rapidly being depleted in portions of the west and midwestern United States. Stream flows are diminishing while water salination and pollution problems are increasing.

The use of renovated municipal wastewater for agriculture and landscape irrigation is gaining popularity as a potential means of conserving supplies of surface and groundwater. In addition, domestic on-site septic systems are being developed which utilize land application of renovated wastewater to lawns as a means of final treatment and disposal. The potential of human disease transmission from the use of municipal wastewater and the disposal of domestically treated wastewater effluent on lawns needs to be assessed. Current public health concern rests on the following observations:

- 1) raw sewage often contains large numbers of pathogenic organisms,
- 2) current treatment practices may not eliminate all of these organisms,
- 3) pathogens may persist in the environment for long periods, and,
- 4) the minimum infective dose to humans may be very small, especially for susceptible people.

In order to satisfy concerns regarding health risks associated with final disposal of treated effluent onto lawn surfaces, it is necessary to establish a means of assessing the risk of disease potential. A general procedure for assessing risk involves identifying the particular hazards, describing the properties of the hazardous substances and making risk estimations based on disease statistics, epidemiological studies or disease transmission models.

Due to the tremendous investment of effort and cost required, few epidemiological studies which directly address the health risk associated with land application of wastewater have been carried out in the human population. It is generally not feasible to perform controlled epidemiological experiments in human populations, due to the logistics required, and the moral and ethical implications; therefore, studies involving humans are often retrospective and may contain many uncontrolled, confounding factors.

A mathematical modeling approach can be used to predict the risk potential when direct monitoring of risk by epidemiological studies and disease statistics is not possible. This approach requires adequate information on pathogen concentrations in wastewater, pathogen survival in the environment and pathogen infectious dose to humans in order to effectively evaluate the potential health risks. A great deal of this information currently exists for bacteria, protozoa and helminths. As shown in the following literature review, however, there is a lack of definitive information on viruses in wastewater. This is primarily due to the fact that methods for quantifying certain viruses in environmental samples are neither accurate nor easy to do. Most importantly, information on the dose-response relationship between viruses in the environment and exposed populations is essentially nonexistent.

The use of a controlled animal population which would mimic the human response in health or disease may serve as an excellent method for providing information on dose-response to viruses in the environment. Although the use of animal models requires the extrapolation of data to humans, careful selection of an appropriate animal model may provide information which gives a good indication of the actual rather than the potential risk. Major decisions that will determine the future of the use of renovated wastewater for irrigation are currently being made by policy makers. Many of these decisions, including limits on allowable pathogen concentrations in wastewater, are being based on information from areas of scientific uncertainty. Use of an effective animal model may be an important tool in providing information which is needed to establish criteria which will adequately protect public health. Data obtained from animal model studies may be useful to professionals from a variety of disciplines, including: epidemiologists and microbiologists researching disease incidence and transmission; engineers attempting to develop technology which will best protect man and the environment, and; environmental scientists and toxicologists interested in promoting and exploring methods of water conservation and preservation and studying the effects of pollutants in the environment and factors effecting persistence.

OBJECTIVES

The purpose of this research was to evaluate the potential for using piglets to assess the risk of viral infection associated with limited direct contact of lawn surfaces irrigated with renovated domestic wastewater. Specifically, the objectives of this research were to:

- * develop a procedure using pigs to evaluate the risk of viral infection associated with surface application of renovated wastewater onto lawns, and,
- * delineate the dose-response relationship to viruses in the environment.

CHAPTER 2

LITERATURE REVIEW

HEALTH HAZARDS ASSOCIATED WITH LAND APPLICATION

Interest in reuse of municipal and domestic wastewater as a means of conserving or augmenting current water supplies, or for final disposal of wastewater has increased in recent years. Wastewater is potentially hazardous to human health because it often contains numerous human pathogens. Many questions concerning the potential for viral disease transmission associated with land disposal of renovated wastewater remain unanswered. Viruses in wastewater present a potential health risk when a number of conditions are satisfied. Initially, they must survive the treatment process, persist in the environment and be present in significant numbers to produce disease in susceptible individuals.

Pathogens in Wastewater

Municipally and domestically renovated wastewater can serve as an important vector for high concentrations of human pathogenic organisms normally associated with enteric diseases (Shuval 1975, 1982, Jones and Watkins 1985). Land application of wastewater may present a risk to human health from infection through ingestion of pathogens, inhalation of contaminated aerosols or transmission through skin (Sorber and Guter 1975, Loehr et al. 1979, Slade 1981, Kaplan 1987). The principal pathogens in sewage include: bacteria, viruses, protozoa and helminths. Bacteria are easily measured; most do not survive well in treatment processes or the environment

and are associated with a high minimum infective dose. Protozoa and helminths, because of their relatively large size, do not survive conventional wastewater treatment in large numbers. The hazards to public health due to bacteria, protozoa and helminths in wastewater are fairly well understood. In contrast, viruses in wastewater have not received much attention, primarily because procedures for environmental recovery and quantification of viruses are not adequate.

Viruses shed in fecal matter are referred to as enteric viruses. Over 100 types of fecally-excreted viruses capable of causing human disease have been identified (Metcalf 1971, Gerba et al. 1975, Rose 1986). Disease-producing enteric viruses in wastewater are listed and classified in Table 2.1.

Poliomyelitis and hepatitis have been the two viral infections most commonly associated with sewage contamination. However, there is a dearth of information concerning hepatitis A virus (HAV), primarily because adequate methods for growing hepatitis A virus have only recently been established (Rose et al. 1986). Epidemiological studies suggest that polio- and hepatitis A viruses are responsible for numerous waterborne epidemics (Shuval 1975). More recently, Norwalk agent and rotavirus from contaminated water have been recognized as a major cause of childhood gastroenteritis and adult diarrhea (Smith and Gerba 1982, Gerba et al. 1984, Rose 1986). Rotaviruses may be present in sewage in lower numbers than the enteroviruses, however, the former may be more resistant and persist for longer periods (Pescod 1988). As with HAV, information on the distribution of rotaviruses in water is lacking, because there is no sensitive method for detecting rotaviruses in environmental samples (Slade 1984). Reoviruses and adenoviruses have also been isolated from treated wastewater, but their disease significance has not been clearly established.

Table 2.1 Enteric viruses potentially present in wastewater and the associated diseases.

VIRUS GROUP	DISEASE
Enterovirus	
Poliovirus	Paralysis, Aseptic meningitis
Coxsackie A and B	Herpangia, pericarditis Respiratory illness, Paralysis
Echovirus	Respiratory infection, Aseptic meningitis, Gastroenteritis
Hepatitis A	Infectious hepatitis
Adenovirus	Respiratory disease, Eye infection, Diarrhea Acute conjunctivitis
Reovirus	Gastroenteritis, Respiratory disease
Rotavirus	Gastroenteritis
Norwalk agent	Nonbacterial gastroenteritis

Enteric viruses are ubiquitous in nature and may spread rapidly by the fecal-oral route in asymptomatic epidemics. Children are most commonly infected because of their antigenic inexperience; natural immunity is acquired with increasing age. In developed countries, where good sanitary conditions exist, the proportion of nonimmunized in the population increases and infection can become more common in older people, in whom symptomatic disease can be more serious (Melnick 1978).

Concentration of viruses in wastewater

In order to evaluate the relative risk of virus disease transmission associated with land application of treated wastewater, it is important to know the concentration of viruses possible in the treated wastewater. Gerba, Wallis and Melnick (1975) reported that enteric viruses can be excreted by infected individuals in concentrations of at least 10^6 particles per gram of feces. The average enteric virus concentration in United States domestic raw sewage, according to Clark (1984), is 500 virus particles/100 milliliters (mL) of sewage. Gerba, Wallis and Melnick (1975) reported finding similar average enteric virus concentrations in domestic sewage (700 viruses per 100 mL), but added that values as high as 46,350 virions/100 mL have been detected. Shuval (1977), reports a slightly higher average enteric virus concentration, stating that domestic sewage may have 10 - 100 enteric viruses per mL of sewage (1000 - 10000 per 100 mL). Slade and Ford (1983) were able to recover enteric viruses in 100% of raw sewage influent samples. They measured enteric virus concentrations up to 9×10^3 plaque forming units (PFU)/L.

Enteric virus concentration in sewage tends to be at a maximum from July until the end of October, according to studies by Buras (1976) and Melnick (1978). Concentrations of rotaviruses in sewage follow a seasonal distribution with values as

high as $>10^5$ particles per liter of untreated wastewater during the winter and spring months (Hejkal et al. 1984, Tyler 1985).

Loehr et al. (1979) pointed out that presumed knowledge of the actual number and concentration of human pathogenic viruses in wastewater is misleading because sampling and analytical procedures are neither standardized nor adequate. The measured concentration of viruses isolated from environmental samples may be 90-99% lower than actual concentrations due to the limitations of virus recovery procedures (Akin et al. 1977).

Effectiveness of Treatment Processes in Virus Removal

Health hazards associated with wastewater-borne enteric viruses are determined primarily by the extent and effectiveness of treatment processes and the ultimate quality of the renovated water. The efficiency of a waste treatment system in the removal of viruses depends on many factors including; treatment process design, nature of effluent and season (Cogger 1988). Varying degrees of treatment are used to lessen the probability that viable pathogens will be released to the environment. Sewage treatment consists of a sequence of processes designed to reduce suspended solids, organics and inorganic nutrients and undesirable biological components. The processes are described as primary, secondary and tertiary treatment.

Primary treatment is a physical process consisting of the removal of large solid materials by sedimentation and/or screening. Secondary treatment involves biological treatment to remove soluble organic matter using microorganisms in activated sludge, trickling filters and oxidation ponds. Tertiary treatment is an advanced treatment using physiochemical processes to further remove sewage components such as nutrients, dissolved organic matter, heavy metals and some bacterial and viral pathogens, by adsorption, filtration and disinfection (Bitton 1980).

The majority of pertinent literature reviews the effectiveness of removal for bacteria and coliphages, with limited information available on some enteric viruses. Unfortunately, data on the removal efficiency of various wastewater treatment methods are often based on laboratory studies which may not accurately mimic what occurs in practice (Foster and Engelbrecht 1973). Several authors have indicated that laboratory experiments may yield misleading information on virus removal due to the increased difficulty of removal of viruses bound to organic material in field situations (Berg 1973b, Akin et al. 1978).

A number of treatment processes actively remove viruses, others do not. In addition to removal, viruses can be inactivated by high pH, by chemical oxidation with disinfectants, by photooxidation with UV light and by ozone (Gerba 1981). The amount of chlorination which would be necessary to significantly reduce virus numbers would likely cause environmental and biological damage and therefore is not a reasonable method for disinfection.

Rose (1986) points out that enteric viruses have been known to survive primary and secondary treatment, as well as chlorination. It is important to bear in mind, when discussing degree of virus removal or survival during wastewater treatment, the health significance of the remaining organisms. For example, if wastewater contains 10^5 cell culture infective doses (CCID)/L, a seemingly impressive 99% removal, or 1% survival, will yield an effluent with 10^3 CCID/L; a level which may still be of public health concern.

Onsite Wastewater Treatment Disposal Systems

Many domestic wastewater treatment systems are now being developed which utilize land application of renovated wastewater to lawns and ornamental gardens

as a means of final treatment and disposal. Approximately one-third of all homes in the United States are served by on-site septic systems (Otis et al. 1980) and it is estimated that one-half million new on-site systems are being built each year (Perkins 1989). In many rural and suburban areas on-site systems are the only economically viable means of wastewater treatment.

Septic systems are soil-based wastewater treatment systems. They typically consist of an underground septic tank and a filter or absorption field. Sewage in the septic tank undergoes anaerobic digestion, resulting in the production of sludge, which settles to the bottom of the tank, and a floating layer of light solids or fats. Wastewater detention time within a septic tank varies from 24-72 hours (Bitton 1980).

The filter or absorption field is designed to distribute effluent to the soil in order to take advantage of absorption and soil infiltration as part of the treatment process. In a typical system, wastewater from the septic tank is applied to the absorption field through a system of perforated pipes surrounded by gravel or crushed stone. Specific soil characteristics such as soil texture, structure, depth and type are important criteria for determining the suitability of this type of disposal system. Insufficient soil depth or structure, and unsatisfactory hydraulic conductivity can limit the adequacy of conventional septic systems for on-site wastewater renovation.

Alternative methods such as low pressure pipe systems, at-grade soil absorption systems and mound systems have been developed. However, all of these methods require a minimum amount of soil for wastewater renovation (Perkins 1989); making use of these systems unacceptable in areas with shallow soils.

The recirculating sand filter system is an example of an alternative system which may be used when soil characteristics are not adequate for other types of wastewater treatment. The system was developed by Hines and Favreau (1975) and consists of a

septic tank, recirculation tank and an open sand filter. The final stage of wastewater treatment (discharge of the effluent) is accomplished by release of wastewater to surface waters or by application of wastewater to land.

Release of wastewater from on-site systems, to aboveground waterways, as a means of final disposal can produce increased oxygen demand, cause increases in nitrogen and phosphorous, and initiate algal growth (Arceivala 1981); processes which could disrupt the ecosystem over time. As an alternative, on-site septic wastewater may be transported to sewage treatment facilities for further treatment, but access to these facilities may not be readily available in rural areas (Scalf and Dunlap 1977).

On-site disposal systems can reduce pathogen concentrations by several orders of magnitude, however, it is impossible to achieve complete, continuous elimination of pathogens. In a laboratory study by Gross and Mitchell (1985) it was determined that enteric viruses loaded onto sand filter columns broke through the filters once the sand became saturated with enteric viruses. The columns were unable to continue to absorb the large numbers of viruses passing over the sand grains. Average filter effluents were 438 PFU/L when the septic tank effluent virus concentration was 1.8 million PFU/L.

The effectiveness of alternative types of on-site systems, as well as systems operated under near-freezing or near-saturated conditions, is not well established (Cogger 1988). Many domestic disposal systems fail within three years as a result of poor design or installation. It is estimated that <40% of existing systems function properly (Cantor 1985). The design life of a septic system is on the order of 10 - 15 years. Failure after 3 years and within the normal design life is likely to be due to poor maintenance (Perkins 1989). Because of significant failure rates, as well as basic

inadequacies in system design, septic systems have tremendous potential to deposit large numbers of virus particles onto lawn surfaces.

Land Application of Wastewater

Human wastes have been applied to soil for centuries as a means for supplying soils with valuable nutrients. As early as 1870, government officials of Massachusetts approved the irrigation of land with sewage (Bitton 1980). Land application makes use of natural physical, chemical and biological properties of soil and may be considered a form of wastewater treatment.

Methods of Application

Three basic methods are used in the application of renovated wastewater to land. They are the slow rate irrigation method, the overland flow method and the rapid infiltration method. The method used depends on the condition and loading rates of the soil and the waste treatment system design.

The irrigation method, or slow-rate land treatment, is used to renovate and reuse water while disposing of waste effluent and providing water for grass or crops. Method of application is via sprinkler or surface application on soils, with soil infiltration at a rate of 1.5 to 10 cm/week (Bitton 1980). Slow-rate systems achieve a high degree of treatment as the wastewater flows through the plant-soil matrix. The low loading rates of this method allow much of the applied wastewater to be lost through evapotranspiration (Thomas 1986). That which is not lost to evapotranspiration contributes to growth of crops and recharge of groundwater.

The overland flow method consists of allowing wastewater to flow for 50-100 m along a 2-8% slope covered in vegetation. The runoff is collected in a ditch at the

bottom of the slope. Less actual wastewater treatment is accomplished with the overland flow method than with the slow-rate irrigation method. The overland method typically returns 50-60% of the applied wastewater as direct surface runoff while only 10% of the water percolates through the soil. This type of system requires a low permeability clay soil or an artificial barrier preventing soil percolation (Small 1978). Loading rates of wastewater to land range from 5 to 14 cm/week (Bitton 1980).

Rapid infiltration systems can utilize a soil loading rate of wastewater exceeding 90 m/yr (Thomas 1986). Wastewater is applied to moderately and highly permeable sandy soils using a series of shallow basins or holding ponds into which wastewater is placed. The system is designed to take advantage of high soil infiltration rates allowing most of the applied wastewater to percolate through the soil (Wright and Rovey 1979). Surface runoff is prevented while up to 99% of the applied wastewater is contributed to ground water as aquifer recharge.

Virus Survival in the Environment

Virus survival in soil and on vegetation has a direct bearing on the probability of transfer of the pathogen to humans. A few studies have been carried out specifically to evaluate the influences of different factors on virus survival, particularly in soil. The factors most frequently cited in affecting virus survival are temperature, relative humidity, soil moisture content, degree of virus adsorption to soil, soil pH and exposure to light. Hurst et al. (1980) stated that temperature and adsorption to soil may be the most important factors affecting virus survival. They concluded that the temperature is a significant predictor of virus survival.

The overall effect of sunlight on virus inactivation is not clearly defined. Virus inactivation may be more efficient during the daylight hours than at night,

however, this may be due to temperature differences. Direct sunlight (uv light) may be a factor affecting virus survival on vegetation but has not been adequately studied (Gerba and Goyal 1983). In one study (Cutchin and Dayhuff 1962), measles virus was inactivated fairly rapidly by exposure to light, but poliovirus was unaffected.

Harper (1961) and Songer (1967) found that recovery of polioviruses in aerosols was reduced with a decrease in relative humidity, indicating a possible dependency of viability on humidity level. Songer (1967) found that different groups of virus exhibited varying sensitivity to relative humidity.

Soil

Viruses generally persist in soil on an average of 8 days, with an approximate maximum survival time of 100 days (Kowal 1982). Wellings et al. (1974) reported poliovirus survival up to 28 days in soil and Feachem (1981) found that poliovirus and coxsackie virus would survive up to 180 days in soil stored at 4°C. Tierney et al. (1977) found that polioviruses survived in soil following irrigation with wastewater for 11 days during the summer and 96 days during the winter. Enteroviruses may be able to survive for prolonged periods of time (70-160 days) under certain soil conditions by being retained in the top layer of soil, where they become inactivated very slowly (Lefler and Kott 1974, Damgaard-Larsen et al. 1977). The rate of inactivation of poliovirus and coxsackie B3 virus in soil was directly related to temperature and moisture (Yeager and O'Brien 1979). Virus infectivity in soil was lost after approximately 12 days at 37°C and 180 days at 4°C in saturated soil. Under low soil moisture conditions viruses persisted in soil for 3 days at 37°C and 30 days at 4°C.

In an examination of virus survival after land application of wastewater in northern Florida, the virus decline in soil was more rapid in warm dry conditions than

in the warm wet season (Bitton et al. 1984). No virus was observed after 8 days under warm dry conditions, while virus persisted for up to 35 days during the wet season, indicating that virus survival is affected by desiccation and temperature.

Vegetation

Virus survival on vegetation is believed to be shorter than survival in soil due to an enhanced exposure to adverse environmental conditions (Loehr et al. 1979, Kowal 1982), although supporting evidence for this claim is not readily available. Poliovirus can survive on crops for up to 3 to 5 weeks after application but a 99% reduction in detectable virus occurred within 5 - 6 days (Larkin et al. 1976, Tierney et al. 1977, Ward and Irving 1987). Rotavirus SA-11 survived on vegetation for 25 days when stored at room temperature, and 30 days when stored at 4°C (Badawy et al. 1985).

In a recent study examining the fate of human enteric viruses on turf grass, Badawy, Rose and Gerba (1990) found that temperature and virus type influenced virus inactivation rates. The study included poliovirus, rotavirus and coliphage seeded onto 260 sq cm plots at different times of the year. During winter conditions (4 - 10°C) 24 hours were needed to achieve 99% (2 log₁₀) virus inactivation. Only 8 - 10 hours were needed to achieve the same reduction under summer conditions (36 - 41°C). As the temperature increased beyond 38°C the rate of virus decay became more dramatic. In an earlier study Brown et al. (1980) found that naturally occurring coliphage on grass treated with sewage sludge were inactivated within 48 hours, but the air temperature was not specified.

Because longer virus survival times have been observed in soil, the recontamination of vegetation from rainsplash may be more of a factor in contamination of vegetation than actual survival of viruses on plants (Lefler and Kott 1974, Dubois et

al. 1976). This is, however, an effect which would be minimized on dense growth such as grasses typical of lawns and golf courses.

Minimal Infective Dose

The degree of risk of infection from viruses in wastewater depends on many factors. The latter include the efficiency of the particular waste treatment process in removing pathogens; the survival of pathogens in the effluent, in soil or on crops; and the minimal infective dose (MID) required to infect humans (Shuval 1975). The MID is generally defined as the dose required to infect 50% of the population (ID_{50}). The number of pathogens which must be ingested by a human to cause infection varies greatly because of the variation in human susceptibility, resistance to disease, type of virus involved and the age and state of immunity of the host (Ward and Akin 1984).

The levels of virus required to produce infection may be very much lower than the number of bacteria which must be ingested to produce infection (Shuval 1975). A single infectious virus unit has been shown to be capable of causing infection in susceptible persons (Plotkin and Katz 1967, Westwood and Sattar 1976). Plotkin and Katz (1967) established experimentally that as little as one tissue culture infectious dose of poliovirus and other enteroviruses is sufficient to infect a percentage of susceptible persons. A more recent study of poliovirus infectivity in 2 month old infants yielded a 50% ID of 72 TCID₅₀ (Minor et al. 1981). In a limited study of the minimum infective dose of ECHO virus for humans, it was determined that at least 100 viral particles are necessary to provoke infection in 50 % of hosts, and that as few as 1 PFU may be capable of infecting 1% of a susceptible population (Schiff et al. 1984). Cliver (1980, 1981), used piglets and porcine enteroviruses (PE) administered orally to establish MID. He reported calculated values of 1800-2500 PFU for porcine enterovirus-

serotype 3 (PE3) and 600-750 PFU for PE7. These values may be unrealistically high, however, because of problems with methodology used to establish infection, and the small data base involved. It may be of importance, however, that the results of his study indicated that the risk of infection appears to cumulate with repeated, small oral doses of virus. This may indicate that there is no absolute minimum dose that must be ingested at a single instance to produce infection.

Summary

Large amounts of viruses are readily recovered from sewage and it is evident from the literature that these viruses are not completely removed from wastewater effluent during treatment. Based on the information available it is not possible to adequately predict the quantity of virus which will be present following treatment, however, it is apparent from the literature that significant numbers of viruses may be present in treated effluent.

The wide range of reported values for virus survival in soil and on vegetation indicate that survival may be dependent on a number of factors. There appears to be some evidence that virus survival is affected by temperature, amount of adsorption to the soil and soil moisture. Under conditions of low temperature and high soil moisture, which appear to be conducive to enteric virus survival, persistence may be in excess of 100 days in soil and 36 days on vegetation. Some of the variability reported in the literature may be simply because sensitivity to environmental factors is an individual characteristic of the virus, suggesting that generalizations cannot be made. The important factor derived from all of the research to date on persistence of viruses in the environment, is that the remaining viruses may still represent a significant number.

These viruses may be capable of producing infection, particularly in susceptible individuals.

Perhaps the real importance of viruses in the environment may be due to their infectivity, not their numbers. In order to effectively evaluate the risk to human health it would be helpful to establish the number of organisms which must be ingested in order to produce infection in humans. The number of viruses which must be ingested by a human to produce infection appear to be very low. Recent studies indicate that 100 viral particles or less may be necessary to establish an infection in a susceptible host (Block and Schwartzbrod 1989). Some of the variability in the reported MID values may be due to the use of small data sets and the inefficiency of the viral assay methods used to quantify dose. It is apparent from the literature, however, that small quantities of viruses are capable of producing infection. There is some indication that the risk of infection may cumulate with small repeated doses.

EVALUATION OF HUMAN HEALTH RISK

The goal of risk characterization or assessment is to make an estimate of the potential human health risk associated with a particular instance or exposure, or a 'worst case' scenario. Policymakers are under increasing public pressure to "do something" in order to protect groundwater supplies and public health. But how restrictive should measures be to protect groundwater and public health? In order to make rational, informed public policy decisions and establish realistic guidelines for water reuse, more and more information is needed.

Scientific risk estimation needs to be based on extrapolation; from past experience, from scenarios of future possibilities, or from analogies of similar events

(Kates 1977). Risk assessment helps to define the type and extent of environmental or human risk. It generally involves hazard identification, risk estimation and social evaluation (Kates 1977, Ehreth and Jutro 1988). The previous section outlined the potential for disease transmission. This section reviews attempts which have been made to quantify this potential in order to produce a risk estimation. Risk estimations are the measurement of the threat potential of the hazard and are often based on modeling efforts of exposure assessment, epidemiological studies of exposed populations or basic research involving human or animal subjects.

Epidemiology

Prospective, or cohort epidemiology provides a direct measurement of risk, measuring the incidence of disease in a population of exposed individuals over time (Evans 1989, Kelsey et al. 1986). Individuals initially free of disease that have been exposed to factors of interest are monitored for response in prospective studies. Incidence rates are evaluated and compared (i.e., the number of new cases of disease per population at risk per unit time).

Greene (1982) points out that epidemiological studies are important for explaining etiological factors, for quantifying risk and for decision making. However, demonstration of disease transmission associated with the use of wastewater for irrigation is not easy. Documented evidence concerning the nature and extent of risk of diseases transferred from wastewater is lacking. Epidemiology has revealed many of the hazards involved with the discharge of sewage and effluents, but it is difficult to obtain specific measures of risk since the magnitude of the exposure is usually not readily available. Few epidemiological studies have been carried out regarding the occurrence of infectious diseases in humans from the land application of wastewater

because of the tremendous effort and cost involved. Such studies would necessarily involve large, probably young, populations which could be easily monitored and controlled.

Demonstration of transmission of viruses has typically been directed at correlation with disease. Disease, however, does not necessarily result from a viral infection, especially if low doses of virus are encountered. Viruses often cause non-apparent or latent infections i.e., an infected person may not display any overt signs of disease. However, the virus may be actively multiplying in the upper respiratory tract and intestine. The resulting large quantities of excreted viruses from subclinically infected people may lead to disease in susceptible persons within the community. The initial impact, therefore, of viruses in a community, may be silent. The usual epidemiological methods are not adequate to detect low level transmission of viral disease because a single virus type may produce a wide variety of disease symptoms not attributable to a single etiological agent, and several different virus types can produce similar symptoms (LaPorte 1986, Kelsey et al. 1986). Therefore, it may be more cogent to attempt to demonstrate infection rather than clinical manifestation of disease. Serology tests measuring antibody production by an animal or human, signify infection rather than disease as the dependent variable. Use of serology to ascertain infection can eliminate any uncertainty associated with the variety of symptoms caused by viruses (Miday 1980). Antibody production can provide an objective measure of response to infection with viruses.

Epidemiological Studies of Wastewater Irrigation

The United States Environmental Protection Agency supported eleven epidemiological studies of health effects associated with disposal of wastewater and

sludge between 1975 and 1985 (Jakubowski 1986). Three of these studies focused on occupational exposure to viruses and bacteria associated with activated sludge wastewater treatment, four included the general population in the vicinity of treatment facilities, three more examined land application of wastewater for agricultural purposes, and one involved farm families utilizing sludge on cropland. Studies of sewage workers were included because of the belief that they would be subjected to the highest exposure to waterborne pathogens.

Clark (1981) reported no significant difference in illness and virus isolation rates in occupational workers compared to a control group. However, workers who had direct contact with spray irrigant (i.e., nozzle cleaners) had antibody titers to viruses which were significantly higher than all other groups. Results from a later study (Clark 1985) indicated an increase in antibody titers to several enteric viruses among inexperienced workers. Langeland (1986), in a review of the literature on infection risks among wastewater workers, concludes that infection risks among sewage workers is minimal. Jakubowski (1986) warns that although most evidence from occupational studies is negative, problems in quantifying exposure and the absence of a clear endpoint for necessary effect, need to be considered.

In a retrospective epidemiological study of kibbutzim in the State of Israel, evidence of the transmission of infection and disease by sewage spray irrigation was detected. In 77 kibbutzim containing more than 36,000 people using wastewater spray irrigation with nondisinfected effluent, there was a 2-4 times increase in incidence of Shigellosis, Salmonellosis and Hepatitis infections, when compared with 130 control kibbutzim (Katzenelson, et al. 1976). However, because of problems associated with incomplete medical records, it is difficult to draw conclusions from their study (Shuval 1982).

A later epidemiological study by Fattal (1986) attempted to overcome the problems with data reporting methods and control pathways of infection. Eleven kibbutzim using wastewater for irrigation had a seasonal two fold excess risk of enteric disease in the 0-4 year old age group during the summer irrigation months compared with nonwastewater irrigation years in the same kibbutz. No excess was found in other age groups. A study carried out in India (Zoeteman 1980) revealed a strong correlation between sickness in farmers and quality of water used for irrigation.

Schwebach and Durand (1988) investigated the health effects of using chlorinated reclaimed wastewater for public park irrigation in Colorado. Using a prospective cohort research design they concluded that there is no evidence of enteric illness associated with use of reclaimed water for irrigation. However, as is the difficulty with this type of epidemiological study, the data were based on subjective information gathered in telephone surveys of individuals using park facilities. Results of this sort are effected by variation in subject recall and differences in the definition of illness.

Model Exposure Assessments

Hutzler (1978) used models to assess the risk of contracting disease as a result of wastewater discharges. He focused on transmission of hepatitis A virus through water. Models were developed to predict the risk of infection from swimming in polluted water and applied to a single disease (Hepatitis). Models of this sort rely heavily on accurate detailed information about biological and environmental processes. It has already been pointed out in this literature review that attendant variability about these processes, as they relate to the risk of infection from viruses, is substantial.

Hutzler points out that a study of disease transmission in controlled animal populations would be the most likely to give reliable results.

Summary

In order to evaluate the potential risk to human health from land application of wastewater the threat potential needs to be quantified. Several attempts have been made to do this, however, no definitive answers exist. Mathematical models used to assess risk of infection are only as good as the information available on the various parameters describing the process. A great deal of variability exists in the literature for these parameters.

Epidemiological studies aimed at examining the potential of disease transmission from land application of wastewater have not provided definitive data at this time. Use of disease statistics may be misleading since enteric viruses often produce subclinical infections. In addition, even when symptoms are reported they may be due to any number of virus types or even a result of other etiological factors which produce similar symptoms.

Epidemiological studies designed to address the potential for disease transmission or viral infection have typically anticipated that occupational workers would be at greatest risk of infection. Most studies have concluded that the risk of disease or infection is low, however, occupational workers may not be the group which would be at greatest risk. The latter is more likely to include children, and old or debilitated persons. It may be that occupational workers have developed immunity to infection, from long term exposure to low levels of viruses. In addition, occupational workers are encouraged to use special precautions to protect themselves from excessive exposure to potentially harmful substances in wastewater. In contrast, the general

population may be exposed to wastewater effluent used to irrigate land without knowledge of its use. While the evidence of disease as a consequence of contact with wastewater used in irrigation is not overwhelming, it may be that the usual epidemiological methods are not adequate for establishing this correlation.

GUIDELINES FOR REUSE OF WATER

It wasn't until the 1940's, with the improvement in pathogen detection and an increased understanding of the process of disease transmission, that the health aspects of land application began to be an issue of concern. In 1918 California developed regulations for the use of renovated wastewater for crop irrigation (Ongerth and Jopling 1977). These regulations were modified and made more stringent in 1933. Other states and developing countries began to copy California's wastewater disposal requirements.

In 1967 California established the Wastewater Reclamation Reuse Law, providing standards relating to applications of wastewater. According to California standards, adequately disinfected wastewater for unrestricted irrigation of parks and playgrounds has a 7 day median coliform concentration which does not exceed 2.2 organisms per 100 milliliters, and a 30 day maximum not in excess of 23 organisms/100 mL. Effluent suitable for landscape irrigation (golf courses, parks, playgrounds) is limited to 23 coliforms/100 mL (Foster and Jopling 1969).

Because viruses in wastewater may be few in number and difficult to isolate, measurement of the nonpathogenic coliform group of bacteria, a normal component of human feces which are easily detected, is used to give an indication of the occurrence and intensity of viruses in treated effluent. However, as an indicator for the safety of

renovated waters they may not always be reliable. The major cause for discrepancy between bacterial indices of pollution and the occurrence of enteric viruses in water has been attributed to the greater survival capability of viruses. Treatment procedures may be more efficient at removing coliform organisms from sewage than they are at removing viruses. Berg (1973a) reported that coliform bacteria were not detected in laboratory tests with seeded effluents filtered through sand after a time when the viruses still persisted. Additionally, enteric viruses have been isolated from samples of groundwater which contained no fecal bacteria (Marzouk et al. 1980, Fattal et al. 1984, Gerba et al. 1984). Replication of coliforms in water can further confuse interpretations of water quality. These factors may lead to an underestimation of the dangers of more resistant microorganisms such as viruses. Perhaps most importantly, there is an apparent lack of correlation between coliform levels in water and the incidence of disease. Therefore, it is unlikely that the absence of coliforms can guarantee that viruses are not present, yet this is typically the standard that is used to establish the acceptability of water reuse. The presence of any viable bacteria in disinfected water suggests that the treatment procedure is not completely adequate since these organisms are usually less resistant to disinfectant than viruses (Berg 1973a).

Arizona is one of the few states which has attempted to regulate the viral concentration of wastewater effluent for irrigation. Current Arizona regulations governing the reuse of wastewater for landscape irrigation include enteric virus standards of 125 PFU/40L (Kramer 1985).

Summary

Guidelines for use of wastewater for irrigation are currently based on epidemiological studies and knowledge of pathogen survival in treatment processes and

in the environment. It is apparent from the literature that clear epidemiological evidence quantifying the health aspects of wastewater does not exist. Additionally, knowledge of pathogens' survival and the efficiency of treatment processes in removing them, as well as our ability to assess the fate of pathogens in the environment is limited. It has been pointed out that the methods for virus detection and enumeration are not adequate for use in establishing guidelines. The guidelines which do exist are based on sketchy epidemiological data and research data often carried out using indicator organisms because of the difficulties encountered in attempting environmental assays of enteric viruses in the environment. The isolation of enteric viruses in waters meeting coliform standards considered adequate to ensure safety from infectious agents suggests the potential for transmission of enteric virus in water meeting the current standards. It is essential to continue establishing and exploring other means for assessing the risk of viral infection from contact with wastewater effluent.

CHAPTER 3

JUSTIFICATION OF THE ANIMAL MODEL

The goal of animal model research is to emulate a human condition in health or disease, i. e., the model closely mimics human structure, function and/or behavior (Philips and Tumbleson 1985). It is paramount to choose an appropriate animal, one which will serve as a useful indicator of the human response being investigated. Leader and Padgett (1980) proposed that animal models should:

- 1) accurately mimic the desired function or disease,
- 2) be available to multiple investigators,
- 3) survive long enough to be functional,
- 4) be handled easily by most investigators,
- 5) fit available animal housing facilities,
- 6) be of sufficient size to provide multiple samples, and,
- 7) be polytocous so that multiple offspring are produced for each gestation.

Use of an animal model of an infectious disease allows for manipulation of the exact moment and route of infection, the quantity and virulence of the organism as well as various environmental factors. Extrapolation of data from animal model to humans is appropriate if the process in the model also occurs in the natural infection process of humans.

THE MODEL

The model system employed in this study for the evaluation of the risk of virus infection associated with surface application of wastewater, was comprised of young weanling swine and their associated porcine enteroviruses. Swine were selected because they have a digestive tract which is analogous to the human digestive system and they are easy to handle and care for. In addition, porcine enteroviruses which infect pigs are similar to human enteroviruses in all aspects. Porcine enteroviruses are known to be 'host specific', that is, they will only cause infection in pigs. These factors combine to make the pig an attractive model to use in assessment of potential human health risk resulting from contact with enteric viruses in wastewater.

This study concerns the evaluation of using pigs to assess the health impact of irrigating private lawns, golf courses, school yards, parks and playgrounds with wastewater. Young children, who might frequent some of these areas, present the greatest health concern, since children have the tendency to roll around in the grass and perhaps even eat the dirt. Young pigs are well known for their ability to 'root' around in the soil, providing a potentially suitable model for this 'worst case' scenario envisioned with children.

Porcine Enterovirus

Eleven serotypes of porcine enteroviruses (PE) have been identified, based on cross-neutralization tests in cell culture (Derbyshire 1989). The porcine enteroviruses are of the family Picornaviridae. They were originally believed to be associated with diseases causing stillborn(S), mummification in the womb(M), embryonic death(ED) and infertility(I). For this reason the porcine enteroviruses are often referred to as

SMEDI viruses. The porcine enteroviruses have been extensively studied and appear to resemble their human counterparts in every aspect (Derbyshire 1981). They are resistant to lipid solvents and relatively heat resistant (Derbyshire 1989). In addition, they are known to be fairly resistant to chemical disinfection; fairly high concentrations of commonly used disinfectants are required to inactivate the porcine enteroviruses (Derbyshire and Brown 1979).

Pathogenesis

The Picornaviridae are small RNA viruses containing the genera enterovirus, cardiovirus, rhinovirus and aphthovirus. The virions are non-enveloped, spherical nucleocapsids, 22-30 nm in diameter. Replication takes place entirely in the cytoplasm (Pensaert 1989).

The picornaviruses are primarily inhabitants of the gut, considered mostly nonpathogenic (Anderson 1981) and are generally species specific. Picornaviruses are ubiquitous in nature; most species of domestic animals are susceptible to one or more of the viruses (Anderson 1981). Singh and Bohl (1972) tested enterovirus excretion by cell culture in 26 pigs from 4 litters and found that all pigs were excreting enteroviruses of at least 6 serotypes by 40-60 days of age.

Transmission of enteroviruses occurs by the fecal-oral route but indirect transmission by materials contaminated by or originating from infected animals also occurs (Derbyshire 1981). However, Anderson (1981) suggested that these viruses may have the potential to first infect the respiratory tract by aerosol and then reach the intestine as a result of viremia. In most cases porcine enterovirus infections are subclinical (i.e., asymptomatic) when the infection occurs post-natally.

Porcine enteroviruses associated with SMEDI disease have little or no pathological effect on the sow. These viruses are pathogenic only when swine are infected during embryonic or fetal life (Wang and Pensaert 1989). Most gilts have been naturally infected with enteroviruses prior to sexual maturity and are, therefore, immune at breeding. Enteroviruses are probably introduced into the farrowing house by carrier sows (Anderson 1981).

Infection occurs by ingestion of porcine enterovirus with initial multiplication taking place in the tonsil, intestine and associated lymph nodes (Derbyshire 1989). Intestinal replication and fecal excretion of virus may persist for up to eight weeks (Derbyshire and Collins 1972), however, Singh et al. (1960) found that after oral challenge with 10^5 plaque forming units, peak virus excretion occurred by 3 days post infection and was not detected after 19 days.

Porcine Immune Response

Animals are continually exposed to microorganisms of many kinds which enter the body through mucous surfaces of the respiratory, digestive or urinogenital tracts, and through the skin. While these organisms may not be present in sufficient quantity to produce observable disease symptoms, antibodies to specific antigens may appear in the serum. An immune response is highly specific, that is, antibodies are produced which react with the particular antigen which induced the immune response, but not usually with other antigens unless they were very closely related. Antibody production can then be detected by laboratory techniques, as a measure of exposure to a microorganism and subsequent infection.

An animal that produces antibody in response to a microorganism is said to be actively immune to that organism. Following injection of an antigenic substance into a

previously uninfected animal a latent period occurs during which antibody is not detectable or does not increase in the circulating blood. This can vary from 5 to 14 days. After the latent period there is a sharp rise in titer with antibody reaching a peak or plateau of varying duration (Carpenter 1965) (Figure 3.1). Antibody will usually appear following antigen exposure in mammals after a minimum interval of 4 days (Brambell 1970). Kelly (1964) found that a 200,000 TCID₅₀ dose of a porcine enterovirus administered orally to 7 pigs produced antibody in all pigs by 7 days after inoculation. Virus neutralizing antibodies have been observed in pig serum within six days of infection, reaching high titers by the 9th-13th day after oral dosing (Baba et al. 1966). Hazlett and Derbyshire (1976) indicated that peak serum levels were reached by 9 days and had diminished significantly within 36 days of dosing. Russell and Edington (1985) concur, stating that the primary immune response usually takes 7 - 10 days to appear.

Antibodies from an immune individual can be transferred to the circulation of another individual giving it a passive immunity, which is immediate, but short lived, persisting only as long as the antibody remains in the host's circulation. Newborn animals lack active immunity and would have little resistance to disease if it weren't for the passive immunity transmitted from mother to offspring. Nursing piglets are often protected against infection by ingestion of maternal neutralizing antibodies in colostrum and milk. Pigs are not born with passive immunity but acquire it by ingesting colostrum during the first few hours of life. Passively-acquired antibodies tide young animals over until they are able to respond to antigens they encounter by themselves (Brambell 1970). Colostrum is maximally absorbed during the first 12 hours of life (Russell and Edington 1985). Transmission of passive immunity in milk to offspring declines rapidly after birth and effectively ceases at about the end of the first day

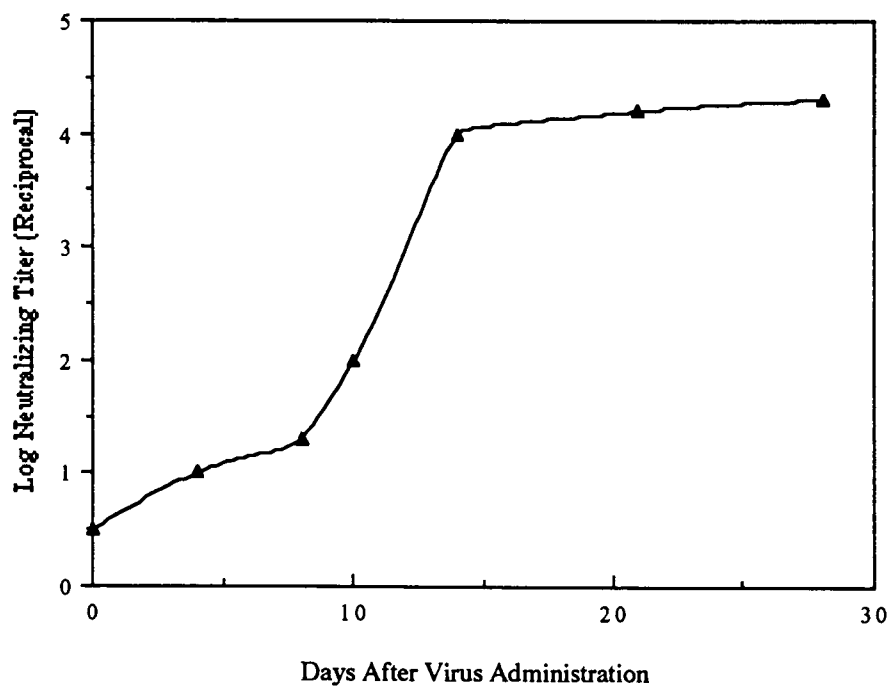


Figure 3.1 Production of antibody during experimental infection of humans with influenza virus [after: B. Murphy et al., In: S. Baron et al., *The Interferon System* (1987)].

(Brambell 1970). The half-life of passively transferred antibody is approximately 23 days (Cremer 1973). Infection is most frequent in piglets during the post weaning period, indicating that some protection occurs until pigs are weaned (Derbyshire 1989). Results from a study by Bridger (1990) indicate that antibody in cows and sows milk appears to delay rather than prevent infection with rotavirus. She speculates that the low levels of maternal antibody in the gut may help to moderate the infection.

The decline in passive immunity and the point in time of development of active immunity in piglets is of importance to this research. It appears that piglets are capable of producing antibodies by about 10 days of age (Wilson 1974). In studies by Hoerlein (1957) and Sterzlet et al. (1960) young pigs inoculated intravenously with enterovirus at 3 - 8 weeks of age were tested for antibody production. Pigs which had been allowed to suckle normally and had been inoculated with enterovirus at 3 weeks of age produced antibody to enterovirus by 10 days post inoculation. The antibody response was, however, stronger in another group of pigs inoculated with enterovirus at 6 weeks of age. Pigs deprived of colostrum at birth showed no measurable antibody response to enterovirus, when inoculated at three weeks of age. However, these same pigs did produce antibody to enterovirus when inoculated a second time at 6 weeks of age. Miller et al. (1962) found that antibody production in piglets was 20 times greater in 6 week old piglets than in 3 week old piglets. Piglets fed colostrum at birth and inoculated at 3 weeks of age with combined diphtheria and tetanus toxoids, seroconverted within 7 days (Segre and Kaeberle 1962). In a study of colostrum deprived piglets orally inoculated with enterovirus at 1 - 5 days of age, serum neutralizing antibodies were detected by 7 days post inoculation (Kelly 1964). Peak enterovirus antibody levels were detected by 3 weeks following oral inoculation. The discrepancy in age at which piglets were observed to produce antibody may partly

reflect differences in the sensitivity of the assay systems used in these studies. Perhaps the nature of the antigen itself is an important factor in the serologic response in newborn piglets. It appears evident from the literature that piglets allowed to suckle normally are immunocompetent by 3 weeks of age. The ability to produce serum antibodies may reach a maximum by 6 weeks of age.

Serologic testing for antibody production gives a measure of the infection rate and is useful for the diagnosis of viral diseases and in epidemiological studies. Diagnosis of porcine enterovirus infection can be based on a rise in titer of virus-neutralizing antibodies between acute and convalescent serum samples or by specific antibody production in young pigs that have not been previously exposed to virus (Derbyshire 1989). This diagnostic criterion is a binary factor (i.e., the presence or absence of antibody indicating infection or no infection).

REVIEW OF RESEARCH

Most of the research involving porcine enteroviruses has been precipitated by a desire to understand the role of viruses in enteric disease. Germfree pigs have been infected experimentally with porcine enteroviruses to trace the course of infection in pigs. Attempts have been made to quantify the amounts of virus present in various tissues at different times (Baba et al. 1966, Kelly 1964, Derbyshire 1974) and to measure cell mediated responses (Brundage 1980). Recently young pigs were used as a model to examine infection by waterborne enteroviruses in drinking water (Cliver 1981).

Very little wastewater reuse research involving animal subjects has been done. Hutzler (1978), who used a predictive model to assess the risk of contracting Hepatitis

as a result of effluent discharges, notes that the study of disease transmission in controlled animal populations may be important for determining reliable results for risk model validation.

Studies of cattle grazing on sewage farms and experiments where raw sewage, treatment plant effluent or sludge was fed to cattle or hogs were reviewed in 1951 by Snyder. He indicated that no adverse effects, such as overt disease, were detected.

SUMMARY

Animal models may be valuable in the search for improved understanding of human disease. Pigs and their associated porcine enteroviruses have typically been used to investigate the nature of enzootic infections on farms, and to study the pathogenesis of the enteroviruses. Because the porcine digestive tract, the associated enteroviruses and the immune response to infection are similar to those of man, the potential exists to use the system as a representative model for human disease and as an indicator of toxins in the environment.

Piglets meet the criteria for a good animal model proposed by Leader and Padgett (1980). Previous studies have indicated that it may be possible to obtain piglets free of antibody to specific enterovirus serotypes. Piglets are immunologically competent by three weeks of age and will produce antibody specific to a porcine enterovirus following exposure and infection. These antibodies can be easily detected in serum using standard diagnostic procedures sensitive enough to detect low levels of antibody production. The model system is made even more attractive by the fact that while the porcine enteroviruses are similar to the human enteroviruses they are species

specific. Therefore, there is no risk of infection to human handlers from exposure to the porcine enteroviruses.

CHAPTER 4

EXPERIMENTAL METHODS AND MATERIALS

The two phases of this research were to: (1) develop a procedure using pigs as a method of assessing risk of viral infection from environmental exposure to wastewater used for irrigation; and (2) examine the nature of the dose-response relationship.

To establish the potential for the animal model, sera from three week old suckling piglets were screened for antibody production to porcine enterovirus (PE) serotypes 1 - 8, using an indirect immunofluorescence technique. Based on the outcome, porcine enterovirus serotype 2 (PE2) was selected for use throughout this research project.

To ensure that a positive response (indicative of infection) would be detectable, known quantities of virus, covering a range of two orders of magnitude, were administered to piglets by gavage tube. Serum from the pigs was monitored to detect production of antibody for two weeks after oral inoculation.

Once the oral dose producing a positive response was established, experiments were designed to expose pigs to virus applied to grass. Moveable isolation pens, divided into four separate sections designed to prevent pig-to-pig contact were placed outdoors on plots of grass. Water containing known concentrations of PE2 was applied to each plot of grass. One pig was placed into each section of the pen. Following 2-hour exposure, pigs were returned to the animal housing facility. Samples of blood were periodically drawn from each of the pigs to monitor antibody production and establish presence or absence of infection.

LABORATORY PROCEDURES

Cells and Cell Cultures

A continuous line of swine testicle (ST) cells was used for growing virus. Cells were grown in Dulbeccos Modified Eagles Medium (DMEM) (Whittaker Bioproducts, Inc., Walkersville, MD) supplemented with 100 U of penicillin per mL, 100 µg of streptomycin per mL, 0.25% glucose, 0.03% L-glutamine and 10% fetal bovine serum (FBS). Cell culture maintenance medium was similar to growth medium except that FBS concentration was reduced to 2%. Cells were removed from their growth surface for subculturing with 0.05% trypsin (1:250 tissue culture grade-SIGMA Chemical Co., St. Louis, MO) in phosphate-buffered saline (PBS).

Virus

Porcine enterovirus serotype 2 (PE2), strain T80, was provided by L. N. D. Potgieter of the University of Tennessee. Seventy-five cm² flasks containing confluent monolayers of ST cells were inoculated with 1 milliliter of virus stock, incubated at 37°C and monitored for cytopathic effects (CPE). Cytopathic effects are morphological indications of cell degeneration, indicating virus multiplication. When CPE reached 90-100%, cells were scraped from the flask surface into the fluid medium. One milliliter aliquots of the virus stock were stored at -70°C until needed.

Virus Quantification

A quantal assay was used to determine the amount of infectious virus present in stock solution. Assay was carried out by the 50% cell culture infective dose (CCID) method. The 50% CCID represents the viral concentration which causes a cytopathic

effect in the cell layer in half of the samples at this concentration, or with a probability of 0.5. Quantal CCID₅₀ methods generally provide a reasonable estimation of the true viral concentration (Block 1989). According to Hill et al. (1971) the expected reproducibility of the quantal assay method is ± 0.3 Log. The validity of the quantal method relies on two essential hypotheses:

- 1) The presence of one virus particle is sufficient to provoke a cytopathic effect (CPE) in cell culture and the absence of destruction implies the absence of virus, and
- 2) Viruses are randomly distributed in the sample, i.e., each virus has the same probability of being found in a given inoculum (Meynell and Meynell 1970).

Calculations to determine the 50% endpoint were carried out using a method described by Behrens and Kaerber (Block and Schwartzbrod 1989). This method was used because the calculations are fairly simple and it allows for the calculation of a standard deviation.

Procedure

Dilutions of the stock virus were carried out to reduce the number of virus particles to a measurable level in order to determine the endpoint dilution. A dilution factor of one tenth was used in order to minimize the number of plates to be inoculated, while maintaining a reasonable level of precision. Dilution medium used throughout the quantification procedure was DMEM supplemented with FBS (10%), streptomycin and penicillin.

For virus quantification assays, ST cells from 3-7 day confluent culture were collected with trypsin, resuspended in growth medium and distributed in 12-well cell culture plates. When confluent monolayers of swine testicle cells had been established, 0.2 mL of serial dilutions of PE2 were inoculated into the well plates. Eight wells were inoculated per dilution. The inoculum was incubated at 37°C for 2-3 hours to allow the viruses to absorb onto cells. Following incubation, medium was removed from each well, taking care not to damage the cell layer. One mL of maintenance medium was added to each well and the plates were incubated at 37°C. The cell layers were observed daily for 5 days, using an inverted light microscope, to detect cell alterations or cytopathic effect.

The outcome of quantal assay, including calculation of the proportion of positive responses is shown in Table 4.1. Using the calculation method described by Behrens and Kaerber, the logarithm of the viral dose causing a cytopathic effect in 50% of the inoculated wells was determined to be:

$$CCID_{50} = 5 \times 10^{4.75 \pm 0.16}.$$

Preparation of Virus for Experiments

Concentrated standard virus was thawed on the day of administration and diluted to the desired level. Dose was determined on the basis of dilution from standard virus suspensions.

Table 4.1. Calculation of the proportion of positive responses from the results of titration of PE2 in liquid medium.

DILUTION	NUMBER OF POSITIVE RESPONSES ^a	<u>PROPORTION OF RESPONSES</u> POSITIVE RESPONSES (h)	COMPLEMENTS (1-h)
10 ⁰	8/8	1	0
10 ⁻¹	8/8	1	0
10 ⁻²	8/8	1	0
10 ⁻³	8/8	1	0
10 ⁻⁴	8/8	1	0
10 ⁻⁵	2/8	.25	.75
10 ⁻⁶	0/8	0	1
10 ⁻⁷	0/8	0	1

^aPositive indicates cytopathic effect observed

SAMPLE ANALYSES

Serology

The Indirect Fluorescent Antibody Technique (IFAT) developed by Coones et al. (1942) was used to determine the development of serum antibody to PE2. This method is based on the specific union of antigen and antibody. The procedure conforms to standards for detection of infection utilized by the Virology Laboratory at the University of Tennessee Veterinary Teaching Hospital. It is an extremely sensitive and rapid technique with important applications in both research and diagnostic laboratories.

The indirect technique is a two step procedure. First, antibody is reacted with antigen and a complex is formed. In the second step the antibody of the first step serves as an antigen and is reacted with fluorescein-labeled species-specific gammaglobulin antibody, resulting in a labeled complex. The preparation is then examined under epi-fluorescence microscopy with UV light. The fluorochrome molecules excited by UV light emit a characteristic apple green fluorescence. Samples were positive when they exhibited characteristic fluorescence similar to that observed in the positive control.

Preparation of Antigen Slides

Antigen slides for use in the IFAT were made with PE2 virus infected cells. Standard PE2 virus preparation containing $5 \times 10^{4.75}$ CCID₅₀ was inoculated into 75 cm² flasks containing confluent swine testicle cells and incubated for 90 minutes. Following incubation the cells were rinsed and covered with maintenance medium. The flasks were then incubated until at least 25% CPE developed. Cells were scraped from the flask and the medium containing cells and virus was decanted and centrifuged for 10 minutes at 1000 rpm. Supernatant was poured off and the cell pellet was suspended in 0.5 mL phosphate-buffered solution (PBS). Small amounts of the resuspended cells were transferred to a clean, dry slide. Ten distinct spots of the suspension were placed on each slide. The slides were air dried and then fixed in acetone for 10 minutes. Prepared slides were stored at -20°C until needed. An IFAT using positive and negative controls was carried out with each batch of slides, to ensure that the slides were adequate for the IFAT.

Immunofluorescent Assay Procedure

Antigen slides were removed from the freezer and allowed to air dry. Cell spots were outlined using a paint pen and sample sera, diluted 1:40 in PBS, were placed on separate smears of the antigen slide. A 1:40 dilution was arbitrarily selected, prior to beginning experimental procedures, to be the critical titer for establishing a positive response. Slides were incubated in a humid chamber at 37°C for 20 minutes, washed three times for three minutes with PBS, rinsed with distilled water and air-dried. Incubation was repeated with rabbit anti-pig IgG conjugated with fluorescein isothiocyanate (ICN Biomedicals Inc., Irvine, CA). Following incubation, slides were again washed with PBS and rinsed with distilled water. Samples were immediately examined by a minimum of two people, at a magnification of 160 in a microscope equipped with a mercury vapor bulb (Zeiss microscope, Carl Zeiss, Inc., West Germany). Negative controls were run concurrently with each assay.

EXPERIMENTAL ANIMALS

Pigs from crossbred sows were obtained from the swine herd of the Knoxville Branch Station of the Tennessee Agricultural Experiment Station. Prior to initiating experimental infection studies it was necessary to evaluate the availability of piglets free of antibody to porcine enteroviruses. Sera collected from twenty seven three-week-old suckling piglets from three different litters were screened for antibody to eight serological types of PE by IFAT at a dilution of 1:20. At three weeks of age all 27 piglets were negative for PE2. Because this virus did not appear to be prevalent in this area, it was selected for use in all subsequent experimental procedures.

Originally, it was hoped that six week old pigs could be used throughout the study. At weaning (six weeks of age), nine pigs randomly selected from the twenty-seven previously tested piglets were transported to the Brehm Animal Science Building on the Agricultural Campus at the University of Tennessee, for use in the first experiment. Prior to challenge with virus, serum was once again obtained and evaluated for antibody production to PE2. At this point all nine piglets had developed titers to PE2 in the three week interval since previous testing. These nine pigs were returned to the experimental farm. It was apparent that piglets needed to be obtained prior to weaning and before they had contact with other piglets. Because of this experience, 3-to-4-week-old piglets were used throughout this study. At this age they are immunologically competent but did not have antibody to PE2.

Animal Housing Facility

During experimental procedures pigs were housed at the Brehm Animal Sciences Building under conditions designed to prevent the possibility of incidental viral transmission. Individual pig cages were constructed of expanded steel mesh welded together and raised 2 feet above the floor. Sets of cages were constructed so that each set consisted of either six or ten individual cages connected together. Sets of cages were separated from each other by a minimum of 1.5 meters.

Piglets were placed in every other cage so that pigs were not housed in adjacent cages, precluding pig-to-pig contact and minimizing the possible transfer of virus from one animal to the other. Mesh lids were strapped onto the cages to prevent animals from jumping out. Entry to each cage was accomplished by removal of the mesh lid.

The housing facility was not temperature controlled so piglets with signs of cold stress during the winter months had heat lamps placed over their cages to keep them

comfortable. The animal housing facility is ventilated to the outside of the building and meets standard airflow requirements for industry.

Animal Care and Feeding

Piglets had access to continuous, on-demand water at all times throughout the experiment. Animals were fed standard creep feed, consisting of pulverized corn, soybean meal, dicalcium phosphate, limestone and salt, and supplemented with vitamins and antibiotics. On-demand creep feeders were checked once per day and refilled as necessary. Floor areas beneath the cages were cleaned and washed down once per day, at feeding. All activities and procedures were carried out by a single caretaker familiar with microbiological techniques. Care was exercised to avoid contact with pigs, once virus exposure had taken place. All control pigs were fed, and the floor beneath the control pigs was cleaned, before feeding virus exposed groups of pigs. All procedures were carried out from lowest dose exposure pigs up through highest dose exposure pigs. These procedures were strictly adhered to in order to minimize the possibility of inadvertently transferring virus from pig to pig via the caretaker.

EXPERIMENTAL PROCEDURES

Oral Infectivity Experiment

This experiment was designed to evaluate antibody production in pigs as a result of infection with a porcine enterovirus. In addition, the purpose was to ascertain if the animal housing facilities and care techniques were adequate to prevent inadvertent pig-to-pig transference of virus following experimental inoculation. A secondary

objective was to confirm the oral infective dose required to elicit antibody response reported by other researchers. Information obtained from this experiment was to be used to help determine the appropriate environmental virus exposure dose to be used in subsequent experiments.

Description

Because of concerns regarding the availability of piglets free of antibody to porcine enterovirus raised by the first attempt at obtaining pigs, blood samples were collected from three litters of piglets comprising a total of 33 three-week-old suckling pigs. Sera from these pigs were serologically tested by IFAT for antibody to PE2 virus at a dilution of 1:20. Following sample analysis, ten piglets were randomly selected from the three litters and transported to the animal facility at the University of Tennessee. Each pig was randomly assigned to one of three doses to be administered. Oral doses consisted of: (1) 0 CCID₅₀ (control dose), (2) 10² CCID₅₀, and (3) 10⁵ CCID₅₀.

Administration of Virus

Virus was administered by intragastric inoculation with a stomach tube. Known quantities of PE2 virus suspended in 5 mL of DMEM were expelled from a syringe into the stomach tube. The tube was flushed with 2 mL of distilled water to ensure complete evacuation of the sample. Animals were challenge inoculated with either 10² CCID₅₀ or 10⁵ CCID₅₀. Control pigs were administered 5 mL of DMEM without virus.

Sample Collection

Two to three cc of blood was withdrawn from the anterior vena cava of piglets by a method described by Mount and Ingram (1971), using a 21 gauge, $1\frac{1}{2}$ inch needle and a 5 mL syringe. Piglets were restrained on their backs in a holder designed for this bleeding technique. The needle was inserted on the right side of the carinaform cartilage of the sternum on an imaginary line drawn from the point of cartilage to the base of the ear. The needle was guided medially, dorsally and caudally into the arch formed by the first two ribs. Blood was withdrawn and transferred to a vacutainer tube.

Samples were collected from orally-inoculated piglets one day prior to inoculation and thereafter at 3, 7, 10 and 13 days post inoculation (dpi). Additionally, blood was collected at weaning (6 weeks of age) from 18 piglets sampled originally and which remained on the sows at the experimental farm.

Sample Analysis

Blood samples were centrifuged at 1000 rpm for 10-15 minutes, to separate serum from other blood components. The serum was collected and analyzed at a 1:40 dilution in PBS by IFAT. Results were recorded as either positive or negative by a minimum of two people. Results of most serum samples were recorded on the day of sampling. If analysis was delayed, sera were refrigerated until IFAT was carried out.

Titration of Positive Samples

Positive samples were also tested by IFAT at dilutions of 1:80 and 1:160 in PBS.

Environmental Exposure Experiments

A series of experiments was designed to evaluate viral infection in piglets by virus delivered in distilled water to grass plots. Additionally, a range of virus concentrations were applied to the plots to evaluate the dose-response relationship in this system. Virus concentrations applied to lawn surfaces were chosen based on results of the oral inoculation experiment and an estimate of virus concentrations which might reasonably be present in renovated wastewater under a variety of conditions.

Description

Four experiments were conducted over a period of one year. The timing for each experiment was based on sow farrowing dates and the availability of animal housing facilities. Sows were farrowed at the experimental station on a quarterly basis, in January, April, July and October. A total of 94 three-to-four-week-old pigs were used throughout the experiments. They were divided among the control groups and groups placed on plots with either 4×10^2 , 4×10^3 , 4×10^4 , or 4×10^5 CCID₅₀ of PE2 virus. Two groups of control animals were included in each experiment so that inadvertent transmission of virus would be recognized. One group of control pigs was placed on a plot which had not been seeded with virus, and the second group of control pigs was held in the housing facility throughout the experiment. The purpose of the indoor set of controls was to evaluate the potential for infection from a source other than controlled purposeful exposure.

Animals were held in the indoor cages for approximately 2-3 days prior to challenge, to allow acclimation. Experiment 2 served as a complete replicate of Experiment 1, and Experiment 4 served as a replicate of Experiment 3. Pigs were held for two hours on virus-seeded and control plots in every experiment.

Pens

Moveable 1.2 m x 1.2 m pens, divided into four 0.6 m x 0.6 m sections, were placed on grass plots. Each 0.6 m x 0.6 m (0.36 m²) section held one pig during treatment. Four pigs, one in each 0.36 m² section, were exposed to the same concentration of virus. Pens were placed as far apart as feasible on level ground in an effort to prevent cross contamination from pen to pen.

Application of Virus

Prior to placing piglets onto the grass plots each section enclosed by pens was watered, using a standard plastic watering can, with distilled water containing the desired virus concentration. Each 0.6 m x 0.6 m section received a total of 4 Liters of water seeded with virus. Sections containing negative control piglets received applications of distilled water only. Four liters of water applied to a 0.6 m x 0.6 m area is approximately equivalent to the application of 12 mm of liquid. This is typical of the amount of effluent which might be expected to be applied daily to a lawn surface by slow-rate sprinkler.

Transportation to the Field Site

Piglets were transported between the housing facility and the experiment site in a manner designed to prevent inadvertent virus transmission among pigs. A handler was assigned to one pig in each treatment. Handlers wore disposable coveralls and gloves, which were changed before handling each pig.

Handlers removed piglets from the animal facility cage prior to exposure and the pen following exposure, and held the pig while it was transported from one site

to the other. Following exposure, the handler placed the piglet into the same cage the piglet originally occupied.

Experiment 1

Nineteen 4-week-old piglets were used in the first experiment. Plots were seeded with either 4×10^3 or 4×10^5 CCID₅₀ as described previously. Four piglets served as negative controls and four piglets each were placed on plots with each virus concentration. Seven pigs remained in their assigned cages in the animal facility at all times to serve as indoor negative controls.

Experiment 2

The first experiment was repeated in February with 19, 4-week-old pigs.

Experiment 3

Twenty eight three-week-old pigs were used in the third experiment, carried out in early May. Based on the results of Experiments 1 and 2, virus concentrations were adjusted in the third experiment. Virus concentrations of 4×10^2 , 4×10^3 and 4×10^4 CCID₅₀ per plot were used in this experiment. Four pigs were placed on plots treated with distilled water only, 8 pigs were placed in plots seeded with 4×10^2 CCID₅₀. Four pigs were placed on plots seeded with 4×10^3 CCID₅₀ viruses and four pigs were placed on plots seeded with 4×10^4 CCID₅₀ viruses. Eight pigs remained indoors.

Experiment 4

Twenty eight 3-week-old pigs were used in the fourth experiment in August. Plots were seeded with 4×10^2 , 4×10^3 , 4×10^4 and 4×10^5 CCID₅₀. Four pigs were placed on plots with each virus concentration except the highest concentration. Eight pigs were placed on plots each seeded with 4×10^5 CCID₅₀ virus to equalize the total number of pigs exposed to each virus concentration throughout all four experiments. Four pigs were placed on plots without virus. Four pigs served as indoor negative controls.

Sample Collection

At the start of each experiment blood samples were collected from the anterior vena cava of each pig. Blood samples were collected again, following exposure to grass plots, and the serum was tested for PE2 virus-specific antibody. Blood samples were collected from pigs at 10 days and 20 days post exposure in Experiments 1 and 2. Experiment 3 was extended to include sampling at 27 days post exposure to ensure detecting seroconversion delayed beyond 21 days after infection. However, because delayed seroconversion did not occur, sampling of pigs 27 days post exposure was not done in Experiment 4. Furthermore, seroconversion was not detected before 10 days post infection. Therefore, Experiment 4 included sampling at 14 and 21 days post exposure only.

Sample Analysis

The IFAT for antibody detection, and indirectly for evaluating the incidence of infection in pigs, was used for all samples collected. Samples were diluted 1:40 in

PBS for the test. Samples were examined under a fluorescence microscope and recorded as either positive or negative by a minimum of two people.

Titration of Positive Samples

Samples which were positive on day 20 or 21 were also analyzed by IFAT at dilutions of 1:80 and 1:160.

Climatological Data

Temperature and relative humidity were monitored on days of environmental exposure by obtaining data from the National Weather Service, Alcoa, Tennessee. The values reported were climatological data recorded at the midpoint in the 2 hour exposure period.

CHAPTER 5

RESULTS

Prior to oral challenge inoculation with virus three litters of pigs were tested to determine the availability of pigs free of antibody to PE2 virus. Serologic examination of 33 three-week-old piglets still suckling revealed that antibody to PE2 was not present. Ten of these piglets were randomly chosen, removed from the sow and moved to the animal facility. They were still free of PE2 antibody immediately prior to oral administration of virus. Eighteen of the 23 piglets remaining on the sow were sampled again at six weeks of age, during normal weaning. All eighteen pigs had antibody titers to PE2 at six weeks of age. This confirmed a preliminary experiment that the critical age for obtaining piglets for use in experiments may be approximately three weeks (data not given).

RESULTS OF THE ORAL INFECTIVITY EXPERIMENT

Serologic Testing

Six pigs inoculated orally with known quantities of PE2 in cell culture medium and three pigs given medium which did not contain virus, were tested for serum PE2 antibody at 3, 7, 10 and 13 days post inoculation. The results are given in Table 5.1.

On the third day following oral administration of virus none of the sera from the 9 pigs had PE2 antibody. By seven days post administration PE2 antibodies were detected in sera from two of the three pigs inoculated with 10^5 CCID₅₀ of virus. No additional pigs had antibody by the tenth day post inoculation, but serum samples

Table 5.1. Serologic^a results following oral administration of PE2.

	Day 3	Day 7	Day 10	Day 13
<u>0 CCID₅₀^{bc}</u>				
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
<u>10² CCID₅₀</u>				
1	-	-	-	+
2	-	-	-	+
3	-	-	-	+
4	-	-	-	+
<u>10⁵ CCID₅₀</u>				
1	-	-	-	+
2	-	+	+	+
3	-	+	+	+

^aIndirect Immunofluorescence performed at a serum dilution of 1:40.

^b50% cell culture infective dose.

^cDose administered orally per pig.

collected from all pigs challenge inoculated with virus thirteen days after oral inoculation had PE2 antibodies. The experiment was terminated after 13 days, when all pigs which had received viruses had seroconverted.

Serum Titration

Serum antibody titration of positive samples from pigs 13 days post inoculation were done at dilutions of 1:64 and 1:80. The results are given on Table 5.2. The sera of four pigs which had received 10^2 CCID₅₀ viruses were negative for antibody by IFAT at 1:64 and 1:80. The sera of three piglets given 10^5 CCID₅₀ of PE2 virus were positive by IFAT for antibody at 1:64 dilution, but were negative at 1:80 dilution.

Table 5.2. Serum antibody titer at day 13 post oral administration of PE2.

	TITER ^a
10^2 CCID ₅₀ ^b	
Pig 1	40
Pig 2	40
Pig 3	40
Pig 4	40
10^5 CCID ₅₀	
Pig 1	64
Pig 2	64
Pig 3	64

^aTiter is the reciprocal of the highest dilution of serum in which antibody can be detected.

^b50% cell culture infective dose; amount of virus administered orally per pig.

RESULTS OF ENVIRONMENTAL EXPOSURE EXPERIMENTS

These experiments were designed so that results could be used to assess the feasibility of using piglets to evaluate the risk of viral infection from contact with virus on lawns. The amount of virus applied to sections of a lawn surface were based on the outcome of the oral infectivity experiment and concentrations of enteric virus in sewage reported in the literature. Because antibody to PE2 virus was easily detected in animals inoculated orally with 10^2 and 10^5 CCID₅₀ virus, virus concentration for the first environmental exposure experiment represented both the average concentration of enteroviruses measured in sewage and the highest concentrations anticipated to occur in sewage.

Prior to using pigs in any experiment, it was necessary to ascertain their immunity to PE2 virus. Sera obtained from all experimental pigs prior to exposure were negative for PE2 antibodies.

Serologic Testing

Experiment 1

Serum samples obtained from pigs ten days after environmental challenge exposure to PE2 virus did not have PE2 antibody. By day 20, however, two of four pigs held on plots seeded with 4×10^5 CCID₅₀ had antibody responses to PE2 (Table 5.3).

Experiment 2

Table 5.4 presents the results of the second environmental virus exposure experiment. Serum from all pigs on day 10 was negative for PE2 antibody. Several sera collected at 20 days post exposure had PE2 antibodies. All four pigs from plots

Table 5.3. Serologic^a results following environmental challenge, Experiment 1.

	Day 10	Day 20
<u>NO EXPOSURE^b</u>		
Pig 1	-	-
Pig 2	-	-
Pig 3	-	-
Pig 4	-	-
Pig 5	-	-
Pig 6	-	-
Pig 7	-	-
<u>0 CCID₅₀^{cd}</u>		
Pig 1	-	-
Pig 2	-	-
Pig 3	-	-
Pig 4	-	-
<u>4 x 10³ CCID₅₀</u>		
Pig 1	-	-
Pig 2	-	-
Pig 3	-	-
Pig 4	-	-
<u>4 x 10⁵ CCID₅₀</u>		
Pig 1	-	-
Pig 2	-	+
Pig 3	-	-
Pig 4	-	+

^aIndirect Immunofluorescence performed at a dilution of 1:40.

^bIndoor control, no outdoor exposure.

^c50% cell culture infective dose.

^dTotal virus dose delivered to grass plots in 4 Liters of distilled water.

Table 5.4. Serologic^a results following environmental challenge, Experiment 2.

	Day 10	Day 20
<u>NO EXPOSURE^b</u>		
Pig 1	-	-
Pig 2	-	-
Pig 3	-	-
Pig 4	-	-
Pig 5	-	-
Pig 6	-	-
<u>0 CCID₅₀^c</u>		
Pig 1	-	-
Pig 2	-	-
Pig 3	-	-
Pig 4	-	+
<u>4 x 10³ CCID₅₀</u>		
Pig 1	-	-
Pig 2	-	-
Pig 3	-	+
Pig 4	-	+
<u>4 x 10⁵ CCID₅₀</u>		
Pig 1	-	+
Pig 2	-	+
Pig 3	-	+
Pig 4	-	+

^aIndirect Immunofluorescence performed at a dilution of 1:40.

^bIndoor control, no outdoor exposure.

^c50% cell culture infective dose.

^dTotal virus dose delivered to grass plots in 4 Liters of distilled water.

seeded with 4×10^5 CCID₅₀ virus were positive for PE2 antibody. Two of the four pigs from plots seeded with 4×10^3 CCID₅₀ virus were also positive for PE2 antibodies by 20 days post exposure. Additionally, one pig from the negative control group placed on plots receiving water only was positive for antibody to PE2 on day 20. This was the only incidence of a positive response recorded for a control animal.

Experiment 3

None of the 28 pigs included in Experiment 3 had detectable antibody titers by 10 days post exposure. By day 20, serum from one pig from a plot seeded with 4×10^4 CCID₅₀ had an antibody response. One week later (day 27 post exposure) this pig was still the only pig which had developed PE2 antibody. Serologic results from all pigs are given in Table 5.5.

Experiment 4

Sampling was delayed in this trial until 14 days after exposure because no positive responses had been detected by day 10 in previous trials. Still, sera from all 28 experimental pigs were negative for PE2 antibody when sampled at day 14. By 21 days post exposure 2 of 4 pigs from plots seeded with 4×10^5 CCID₅₀ virus had developed PE2 antibody and 1 pig of 8 from plots seeded with 4×10^4 CCID₅₀ virus was positive for antibody. Sera collected 21 days post exposure from all other pigs were negative. These results are listed in Table 5.6.

Serum Titration

The titers of antibodies in 20 to 21-day post infection pig serum samples are given in Tables 5.7-5.10. Antibody titer was 80 in serum from two positive pigs in

Table 5.5. Serologic^a results following environmental challenge, Experiment 3.

	Day 10	Day 20	Day 27
<u>NO EXPOSURE^b</u>			
Pig 1	-	-	-
Pig 2	-	-	-
Pig 3	-	-	-
Pig 4	-	-	-
Pig 5	-	-	-
Pig 6	-	-	-
Pig 7	-	-	-
<u>0 CCID₅₀^{cd}</u>			
Pig 1	-	-	-
Pig 2	-	-	-
Pig 3	-	-	-
Pig 4	-	-	-
<u>4 x 10² CCID₅₀</u>			
Pig 1	-	-	-
Pig 2	-	-	-
Pig 3	-	-	-
Pig 4	-	-	-
Pig 5	-	-	-
Pig 6	-	-	-
Pig 7	-	-	-
Pig 8	-	-	-
<u>4 x 10³ CCID₅₀</u>			
Pig 1	-	-	-
Pig 2	-	-	-
Pig 3	-	-	-
Pig 4	-	-	-
<u>4 x 10⁴ CCID₅₀</u>			
Pig 1	-	-	-
Pig 2	-	+	+
Pig 3	-	-	-
Pig 4	-	-	-

^aIndirect Immunofluorescence performed at a dilution of 1:40.

^bIndoor control, no outdoor exposure.

^c50% cell culture infective dose; Total virus dose delivered to grass plots in 4 Liters of distilled water.

Table 5.6. Serologic^a results following environmental challenge, Experiment 4.

	Day 14	Day 21
NO EXPOSURE^b		
Pig 1	-	-
Pig 2	-	-
Pig 3	-	-
Pig 4	-	-
0 CCID₅₀^{cd}		
Pig 1	-	-
Pig 2	-	-
Pig 3	-	-
Pig 4	-	-
4 x 10² CCID₅₀		
Pig 1	-	-
Pig 2	-	-
Pig 3	-	-
Pig 4	-	-
4 x 10³ CCID₅₀		
Pig 1	-	-
Pig 2	-	-
Pig 3	-	-
Pig 4	-	-
4 x 10⁴ CCID₅₀		
Pig 1	-	-
Pig 2	-	-
Pig 3	-	-
Pig 4	-	+
Pig 5	-	-
Pig 6	-	-
Pig 7	-	-
Pig 8	-	-
4 x 10⁵ CCID₅₀		
Pig 1	-	+
Pig 2	-	+
Pig 3	-	-
Pig 4	-	-

^aIndirect Immunofluorescence performed at a dilution of 1:40.

^bIndoor control, no outdoor exposure.

^c50% cell culture infective dose; Total virus dose delivered to grass plots in 4 Liters of distilled water.

Experiment 1 (Table 5.7). Sera from all other pigs remained negative for antibodies throughout this experiment.

Table 5.7. Serum antibody titer at 20 dpe^a, Experiment 1.

	TITER ^b
<u>4 x 10⁵ CCID₅₀^c</u>	
Pig 2	80
Pig 4	80

^aDays post exposure.

^bTiter is the reciprocal of the highest dilution of serum in which antibody can be detected.

^c50% cell culture infective dose; amount of virus seeded on plot in which pig was placed.

Results of antibody titration of 20-day post exposure sera in Experiment 2 are given in Table 5.8. All antibody-positive pigs had a serum antibody titer of 40 except one pig from a plot seeded with the highest virus concentration. The antibody titer in serum from the latter pig was 80.

The 20-day post exposure serum from the antibody positive pig in Experiment 3 had an antibody titer of 80. Antibody titration of this sample is given in Table 5.9.

Results of antibody titration of 21-days post exposure sera from antibody positive pigs in Experiment 4 are given in Table 5.10. Serum from the only antibody-positive pig from a plot seeded with 4 x 10⁴ CCID₅₀ virus was negative at serum dilutions greater than 40. One of two antibody-positive pigs from a plot seeded with 4 x 10⁵ CCID₅₀ virus had an antibody titer of 80. The second antibody-positive pig from the plot seeded with 4 x 10⁴ CCID₅₀ virus had a serum antibody titer of 160. This was the highest antibody titer obtained throughout the experimental process.

Table 5.8. Serum antibody titer at 20 dpe^a, Experiment 2.

TITER ^b	
<u>0 CCID₅₀^c</u>	
pig 4	40
<u>4 x 10³ CCID₅₀</u>	
Pig 3	40
Pig 4	40
<u>4 x 10⁵ CCID₅₀</u>	
Pig 1	40
Pig 2	40
Pig 3	40
Pig 4	80

^aDays post exposure.

^bTiter is the reciprocal of the highest dilution of serum in which antibody can be detected.

^c50% cell culture infective dose; amount of virus seeded on plot in which pig was placed.

Table 5.9. Serum antibody titer at 20 dpe^a, Experiment 3.

TITER ^b	
<u>4 x 10⁴ CCID₅₀^c</u>	
Pig 2	80

^aDays post exposure.

^bTiter is the reciprocal of the highest dilution of serum in which antibody can be detected.

^c50% cell culture infective dose; amount of virus seeded on plot in which pig was placed.

Table 5.10. Serum antibody titer at 21 dpe^a, Experiment 4.

TITER ^b	
<u>4 x 10⁴ CCID₅₀^c</u>	
Pig 4	40
<u>4 x 10⁵ CCID₅₀</u>	
Pig 1	160
Pig 2	80

^aDays post exposure.

^bTiter is the reciprocal of the highest dilution of serum in which antibody can be detected.

^c50% cell culture infective dose; amount of virus seeded on plot in which pig was placed.

Climatological Data

The recorded climatological data for all exposure trials are presented in Table 5.11.

Statistical Summary

Results of the environmental challenge experiments are summarized in Table 5.12. A total of 52 pigs were exposed to virus concentrations from 4×10^2 to 4×10^5 CCID₅₀ of PE2 per 0.36 m² of soil surface. To give an idea of the disease incidence as a result of a single exposure to virus, the incidence rate (IR) was calculated. The general formula for incidence rate is:

$$IR = \frac{\text{number of animals with infection}}{\text{total number of animals exposed to risk}} * 100.$$

Table 5.11. Temperature (T), Relative Humidity (RH) and general description of conditions recorded during each environmental exposure experiment.

EXPERIMENT	T (°C)	RH (%)	CONDITIONS
1	4.4	82	Overcast
2	7.8	89	Overcast
3	20	47	Clear
4	27.2	74	Clear

Table 5.12. Number of pigs infected per number of pigs exposed to grass plots seeded with porcine enterovirus for each experiment.

<u>EXPERIMENT</u>	<u>DOSE (CCID₅₀)^a</u>				
	<u>0^b</u>	<u>4 x 10²</u>	<u>4 x 10³</u>	<u>4 x 10⁴</u>	<u>4 x 10⁵</u>
1	0/4	- ^c	0/4	-	2/4
2	1/4	-	2/4	-	4/4
3	0/4	0/8	0/4	1/4	-
4	0/4	0/4	0/4	1/8	2/4
TOTALS	1/16	0/12	2/16	2/12	8/12

^a50% cell culture infective dose.

^bTotal virus dose delivered to grass plot in 4 Liters of distilled water.

^cDose not used in experiment.

The calculated IR for each virus concentration is shown on Table 5.13 and ranges from 0, at a virus concentration of 4 x 10² CCID₅₀, to 66.7, at a virus concentration of 4 x 10⁵ CCID₅₀. The IR versus the concentration of virus applied to 0.36 m² of grass is graphically depicted on Figure 5.1.

Table 5.13. Infection incidence rate in pigs exposed to porcine enterovirus seeded on 0.36 m² of grass.

DOSE (CCID ₅₀) ^{a,b}	INCIDENCE RATE ^c
0 (indoor control)	0
0 (outdoor control)	6.3
4 x 10 ²	0
4 x 10 ³	12.5
4 x 10 ⁴	16.7
4 x 10 ⁵	66.7

^a50% cell culture infective dose

^bTotal virus dose delivered to grass plot in 4 Liters of distilled water

^cNumber of pigs infected per number of pigs exposed to a virus dose, multiplied by 100.

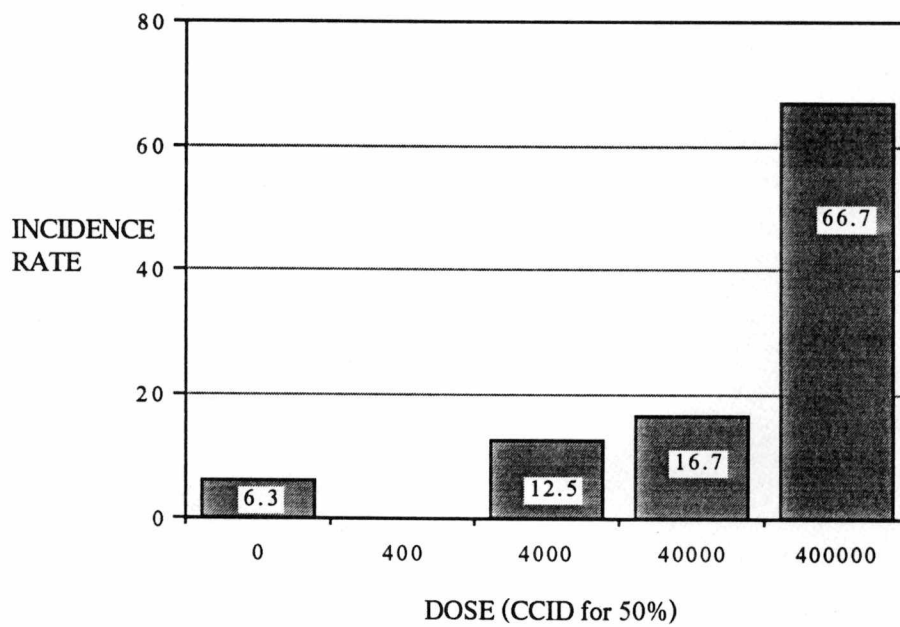


Figure 5.1 Incidence rate of infection in pigs placed on grass plotes seeded with virus.

Statistical analyses of data were performed using the Categorical Data Model (CATMOD) procedure of SAS (SAS 1988). The response of pigs exposed to seeded plots at each dose was compared to the response of non-exposed pigs in order to assess whether the response could be attributed to chance alone. Using contrasts of maximum likelihood estimates the incidence of infection in pigs exposed to grass plots seeded with 4×10^5 CCID₅₀ virus was significant with a probability of 0.005. Comparisons of 4×10^4 , 4×10^3 and 4×10^2 CCID₅₀ with the non-exposed pigs were not significant.

CHAPTER 6

DISCUSSION

ORAL INFECTIVITY EXPERIMENT

Serology

Data from the oral infectivity experiment are consistent with the anticipated immunological response in pigs inoculated with enterovirus reported by other researchers. Serum antibodies are typically detected in pigs orally challenged with enterovirus after about the 6th day (Kelly 1964). Antibody titers generally do not plateau and begin to decline until approximately 21 days post infection (Cartwright and Huck 1963). All pigs orally inoculated with PE2 in this experiment produced serum antibody to PE2 by 13 days post inoculation. The experiment was discontinued at this point since the objective of the oral infectivity experiment was met at this time. Based on what is known of the immune response it can be speculated that additional blood samples collected from pigs 20-21 days post inoculation would be likely to have serum antibody titers substantially higher than those measured at 13 days in this experiment.

The outcome of the oral infectivity experiment established that the IFAT method of antibody detection was a sensitive method to use. Antibody produced in pigs in response to orally administered doses as low as 100 CCID₅₀ of PE2 was readily measured by IFAT.

Minimal Infective Dose (MID)

The oral infectivity results confirmed that infection occurs in 100% of pigs, with an oral inoculation of 100 CCID₅₀ PE2. This is in keeping with previously cited data suggesting that the minimal infective dose of enterovirus in humans is around 100 infectious viral particles or less (Schiff 1984). Minimal infective doses have been calculated for two porcine enteroviruses, using pigs inoculated orally with 1000 PFU (Cliver 1981). An MID₅₀ of 1800-2500 PFU was calculated for PE3 and an MID₅₀ of 600-750 PFU was calculated for PE7. The criterion used to establish that infection had occurred was the recovery of virus from feces in cell culture. Feces were collected only on days 7 and 14 post challenge and not daily. Using this method of detection it is possible that animals which were infected were missed and recorded as negative for virus production. It has been well established that virus particles are not continuously shed in feces during infection. Pigs challenged with porcine enteroviruses intracerebrally, orally and intranasally began to excrete virus particles continuously in feces 1-2 days post challenge (Cartwright and Huck 1963, Kelly 1964). Thereafter virus excretion was intermittent and declined rapidly with the establishment of neutralizing antibodies. In addition to use of a detection method which may have incorrectly resulted in classification of infected pigs as negative, fecal samples were occasionally partially dried by the time of collection and were stored at -20°C for an unspecified length of time until assay was carried out. The effects of desiccation and storage at -20°C instead of -70°C, the temperature generally recommended for virus sample storage, may have resulted in a decrease in virus infectivity by the time of analysis (Berg et al. 1980, Dahling and Wright 1984, Russell and Edington 1985). Blood samples were collected from a few of the pigs 14 days after inoculation in order to evaluate serum antibody production. Cliver used the method of serum-virus

neutralization to detect serum antibodies, at serum dilutions of 1:20, 1:40 and 1:80. He was not able to consistently detect serum antibody in pigs excreting virus. The dilutions used may have been too great to detect a virus neutralizing antibody response. All of these factors may have contributed to skewing the data, because an unknown number of false negative responses may have been recorded. It is very likely that the calculated minimum infective dose, which was based on the number of pigs infected at 1000 PFU, would have been lower and more in keeping with the results of this research, had the techniques used for establishing infection been more effective.

The differences in the MID calculated in Cliver's study and the concentration of PE2 virus capable of establishing infection in 100% of pigs in this research point out the difficulties in comparing results when non-standardized techniques are used to establish the same parameter. In addition to differences in sensitivity of measurement techniques, discrepancies among minimum infective dose estimates may also be related to the strain and isolate of virus used in each experiment. It is possible that the minimum infective dose of PE2 is lower than the minimum infective dose of either PE3 or PE7. It may not be reasonable to assume that all enteroviruses are equally efficient at producing infection. The different assay techniques used to quantify virus in the inoculum may also contribute to the discrepancies in measured MIDs. The amount of virus in an inoculum is not measured as precisely by the CCID₅₀ assay as by the plaquing procedure, and may contribute to some of the variability in reported minimum infective doses of porcine enteroviruses of pigs.

The objective of the oral infectivity experiment was to evaluate the potential for use of pigs and their associated porcine enteroviruses to measure the incidence rate of infection from exposure to grass plots seeded with virus. It was necessary to establish that antibodies produced in response to ingestion of virus could be measured by IFAT.

The doses used to inoculate pigs were not selected with the objective of establishing the MID. They were selected in order to examine the sensitivity of the detection methods, so that concentrations of virus seeded to grass needed to produce infection in pigs could be estimated. Data from this experiment firmly established that ingestion by pigs of as few as 100 CCID₅₀ of PE2 consistently induced an antibody response, detectable by the IFAT at a 1:40 serum dilution, 13 days post inoculation.

ENVIRONMENTAL EXPOSURE EXPERIMENTS

Prevention of Infection in Non-Exposed Piglets

Cross infection of pigs among treatment groups was a concern when experiments were designed, although it had not presented a problem in the oral infectivity experiment. Of 41 piglets serving as negative controls throughout the environmental exposure experiments, only one became infected by the end of the experiment. Experimental procedures were always carried out with all control pigs before exposing any pigs to virus. This precaution may have helped to prevent inadvertent infection of control pigs. It is not known how the one control pig became infected during Experiment 2. It can be speculated that because seroconversion was not detected by IFAT until 20 days post exposure, virus was most likely transmitted to the pig via caretaker, or by any one of the mice, birds or insects inhabiting the animal housing facility. Because infection of a control pig occurred only one time, the facilities used to house pigs during the experiments and the precautions taken to keep the pigs separated were judged to be adequate to prevent inadvertent infection. Within the dose range of this study no difficulty with cross-contamination was evident.

Antibody Response in Pigs Exposed to Seeded Grass Plots

Pigs exposed to grass plots seeded with the upper limit virus dose of 4×10^5 CCID₅₀ readily produced an antibody response detectable by 20 days post exposure. It is likely that the antibody response was not detected earlier because of relatively low antibody response to the virus concentrations to which pigs were exposed. Increasing the amount of virus exposure dose will yield higher antibody titers up to a certain limit (Carpenter 1965). Antibodies to PE2 might have been detected earlier and titers may have risen higher had the pigs in these experiments been exposed to higher concentrations of virus. The relatively low antibody response to PE2 observed in pigs may also be related to the strain and isolate of virus used. The porcine enterovirus used in these experiments may have a low virulence which elicits only a low antibody response.

In spite of the low antibody titers it was clear that observable antibody was produced in pigs in response to exposure to virus seeded on grass. Higher concentrations of virus were not used because the objective of these experiments was to determine whether or not pigs might prove to be a useful animal model for evaluating risks of viral infection from lawns treated with wastewater. It was necessary at this time to use virus concentrations which might realistically be encountered in treated wastewater applied to lawns.

Piglets obtained from the experimental farm at 3 weeks of age were consistently negative for serum antibody to PE2, but often had serum antibody titers for PE1, 3-8. A similar finding was reported by Bridger (1990) when examining rotavirus occurrence in pigs. Of four types of rotavirus groups (A, B, C, and E) antibody to groups A, B and C was present in over 50% of porcine sera collected from pigs aged 3-8 weeks.

Antibody to group E was rarely present in sera from this age group, however, after weaning more than 50% of piglets had antibody to rotavirus E.

Physical Factors Potentially Effecting Incidence Rate

Although it was not the objective of this research to address the effect of physical phenomena on the incidence rate of infection in pigs, the variability of infection among experiments provoked a number of questions that should be addressed. Several physical factors may be contributing to the variability observed among experiments. These may include temperature, sunlight, relative humidity and soil moisture.

The temperature, relative humidity and amount of cloud cover on the days pigs were exposed to concentrations of virus seeded on lawns during experiments 1 and 2 were very similar. During Experiment 1 the temperature was 4.4°C with 82% relative humidity. During Experiment 2 the temperature was 7.8°C and the humidity was 89%. Cool, humid weather combined with overcast skies are optimal conditions for virus survival (Hyslop 1972). A higher rate of infection occurred among pigs at all virus concentrations during the second experiment. The only significant difference between the two experiments was the amount of rainfall immediately prior to the experiment. Although the rainfall amount was not directly measured, it was necessary to delay Experiment 2 for 4 days, because of rain. On the day the pigs were placed on the irrigated plots the rain stopped just long enough to complete the experiment. The increased incidence of virus infection in Experiment 2 may have been related to increased soil moisture, since all other factors were similar between experiments. Evidence exists that decreased rates of virus inactivation may be related to increased soil moisture, but results are variable (Yeager and O'Brien 1979, Hurst et al. 1980).

Perhaps the increased soil moisture in combination with the cool temperature and high humidity during Experiment 2 did help decrease the rate of virus inactivation in soil and on vegetation. It is more likely, however, that the increased incidence of virus infection in pigs in Experiment 2 was simply due to the fact that pigs were able to completely dig up the wet soil during the two hour exposure. This would have increased the probability of ingesting large quantities of soil particles. Adsorption of virus to soil particles significantly prolongs virus survival and immobilizes them in the upper portion of the soil layer (Hurst et al. 1980). Viruses adsorbed to soil particles retain their infectivity (Bitton 1980).

Temperature is perhaps the most important factor in determining virus survival in the environment (Hurst et al. 1980, Badawy et al. 1990). Temperature differences in this study were the greatest between Experiment 1 and Experiment 4. The temperature during Experiment 1 was 4.4°C and during Experiment 4 was 27.2°C. No temperature effect was observed. Two of four pigs placed on plots seeded with 4×10^5 CCID₅₀ became infected during each experiment. It may be that the difference in temperature was not sufficient to significantly alter the concentration of PE2 on grass during the short exposure time. Most laboratory virus inactivation studies reported in the literature were done at temperatures substantially higher than any temperature recorded during these experiments (Yeager and O'Brien 1979, Scheuerman et al. 1991). However, Badawy et al. (1990) measured a 40% reduction in poliovirus concentration on grass at 23°C over a 2 hour time period. The same virus measured on grass during a 2 hour exposure time when the temperature was 10°C was reduced by approximately 25%. Perhaps the data set used in this research was simply too small to determine any real differences in infectivity at different temperatures.

There has been some speculation that virus infectivity in the environment is adversely effected by exposure to sunlight (Frankenberger 1983). It was not evident from this study that sunlight was an important factor in virus decay. During Experiment 4 some of the pigs were placed in shaded areas while the remainder were placed in pens receiving full sunlight. The experiment was not designed to examine the effect of light on virus infectivity. However, because of space constraints 4 pigs exposed to 4×10^4 CCID₅₀ PE2 were placed in shade. Four other pigs exposed to the same virus concentration were placed in sunlight. Only one pig of the eight exposed to grass seeded with 4×10^4 CCID₅₀ PE2 seroconverted. This pig had been placed in a pen receiving full, direct sunlight. All other pigs exposed to 4×10^4 CCID₅₀ PE2 on grass remained negative for PE2 antibodies.

THE MODEL

The animal model met the criteria for a good model as presented by Leader and Padgett (1980). Advantages and disadvantages of the model are listed in Table 6.1. Pigs free of antibody to at least one of the porcine enterovirus serotypes at three weeks of age were readily available from a local farm. Three-week-old piglets were easy to handle and had no difficulty in going directly from sows milk to solid 'creep' feed.

Serologic testing by IFAT gave rapid results and provided an objective measure of infection. Laboratory procedures were simple and can be applied in a standardized manner. The IFAT identified the specific pathogen of interest, eliminating any possibility of incorrectly attributing infection to any number of different pathogens which produce similar disease symptoms. Equipment needed to perform the serologic tests can normally be found in any laboratory equipped to do immunological assays.

Table 6.1 Advantages and disadvantages of the animal model.

ADVANTAGES	DISADVANTAGES
• Simple Laboratory Procedures • Immediate Results • Objective Measure of Infection • Minimum Discomfort To Animal • Can Return Animal To Farm • Can Control Conditions Of Experiments • Can Make Risk Estimates	• Need Pigs Free Of Antibody • Space Requirements • Require Controlled Access Facility • Extrapolation Of Risk To Human

One of the important concerns associated with the use of an animal model is the moral stigma attached to use of animals in science. The procedures outlined in this research were carried out with a minimum of discomfort to the pig. The virus concentrations used were not sufficient to produce disease symptoms in any of the pigs challenged orally with virus or exposed to grass plots seeded with virus. At the completion of all experiments it was possible to return the pigs to the farm, since it had been determined that pigs which remained on the farm normally became infected with PE2 at weaning.

The model provided a means for estimating the risk of viral infection. By monitoring infection in groups of pigs exposed to virus seeded plots it was possible to establish incidence rates of infection. Incidence rates express the probability of

infection per pig exposed to a known concentration of virus applied to a known area of grass. Incidence Rates are used to define the dose-response relationship, which is the basis for quantitative risk estimates between virus concentration on grass and host infection. Although antibody response was not determined to be statistically significant until 4×10^5 CCID₅₀, the bar graph of the data gives a good indication of the trend of the incidence rate. Theoretically there is a low concentration of virus which will not infect any pigs, based on the need for a minimum dose to produce infection, and a high dose which will produce infection in all pigs. Defining the actual dose-response relationship will provide information which will be useful for policy makers charged with establishing an acceptable level of risk. These guidelines must be based on scientific information and balanced with social evaluation of risk. Social evaluation of the risk estimation determines whether or not a risk is acceptable, by describing the consequences of risk and determining the importance of risk (Fischhoff et al. 1979).

Drawbacks to the animal model may include the need to use pigs free of antibody. This was not found to be a problem in this research, but may not be the case in all situations. Requirements for successful housing of animals in a manner which prevents cross contamination may be prohibitive. The general lack of infection among negative control pigs indicated, however, that no special precautions, other than prevention of pig-to-pig contact, and separation of sets of cages containing infected pigs was required to prevent incidental infection. Controlled access to the facility is recommended. In addition to housing requirements, exposure to virus seeded on grass needs to be carried out in areas which have not been recently exposed to swine or swine manure. This precaution will preclude the possibility of virus contamination from another source. The area must be large enough to allow multiple exposures without necessitating the reuse of any one area.

With any animal model there is the problem of extrapolation of conclusions to humans. Use of pigs in this research provided an excellent representation of possible 'worst case' human contact with viruses in water used for irrigation. In addition, the pig is an established appropriate model for gastrointestinal and immunological research because of well demonstrated similarities to the human condition (Philips and Tumbleson 1985).

CHAPTER 7

SUMMARY AND CONCLUSIONS

SUMMARY

A study was initiated in order to investigate the feasibility of using an animal model to assess the risk of viral infection in humans from surface application of treated wastewater onto lawns. The primary objective was to develop a procedure for evaluating this risk of viral infection using pigs exposed to a porcine enterovirus under conditions which simulate potential exposure to virus for humans. Definitive methods for assessing this risk do not exist. Epidemiological evidence for human health risk from viruses in wastewater is sparse. The incentive to develop this methodology was to provide a direct means of assessing the uncertainty of health risk due to the presence of disease causing human enteric viruses in wastewater.

To complete the objectives of this research a combination of controlled oral challenge inoculation experiments and grass plot exposure experiments were done to investigate porcine enterovirus infection in pigs. The availability of susceptible pigs, antibody response of pigs to challenge inoculation with various amount of virus and the efficiency of serology by IFAT to monitor virus infection in pigs was investigated.

Blood samples were obtained from three-week-old piglets from several litters to establish the availability of porcine enterovirus seronegative piglets. Serologic results indicated that many pigs were free of antibody to porcine enterovirus serotype 2 at three weeks of age. Serum samples obtained from the same piglets at weaning had antibody titers to porcine enterovirus 2. This result reaffirmed the ubiquitous nature of the

porcine enteroviruses and the necessity of obtaining piglets by three weeks of age for use in experiments requiring serologic evaluation.

It was established that infection is probable when as little as 100 CCID₅₀ of PE2 is administered to pigs intragastrically by stomach tube. In order to evaluate the potential for pig infection by PE2 from grass seeded with the virus, a series of tests were done using a range of known quantities of PE2 applied to 0.36 m² plots of lawn. The plots were seeded with concentrations of virus which may be present in treated wastewater. Results were statistically analyzed using a categorical modeling program available on SAS (1988). It was concluded from the latter analysis that the relationship between infection incidence rate and virus concentration of the inoculum was not an independent event when doses of 4×10^5 CCID₅₀ virus were used. A single 2-hour exposure of pigs to 4×10^5 CCID₅₀ of PE2 applied to lawns has a potential infection incidence rate of 66.7. The incidence rate dropped off rapidly as the virus concentration seeded onto plots decreased. An incidence rate of 16.7 was calculated for pigs exposed to 4×10^4 CCID₅₀ virus and 12.5 for pigs exposed to 4×10^3 CCID₅₀ virus. None of the pigs exposed to grass plots seeded with 4×10^2 CCID₅₀ became infected.

The animal model proved to be a simple, efficient method for evaluating risk of viral infection to humans who may come in contact with virus in wastewater applied to lawns. The information gained from using this model will be important for public health officials who must determine acceptable guidelines for use of treated wastewater; environmental scientists and toxicologists interested in protection of water resources and fate of pollutants in the environment; and engineers and sanitarians interested in designing effective treatment systems and applying satisfactory management practices. Decisions made regarding human health, conservation of water resources, investment

in water improvement or regulation of water reuse need to be based on sound scientific information. Pigs can be used to provide a useful indication of the risk of viral infection and help to establish best practicable management procedures which will limit potential health and environmental effects.

CONCLUSIONS

The animal model was successful in providing a means for assessing human risk of viral infection from contact with wastewater applied to lawns. The following conclusions were derived from the research experiments.

1. Pigs can be effectively used to assess the risk of viral infection from contact with lawns irrigated with treated wastewater.
 - A. Pigs produce observable antibodies to ingested virus,
 - B. Reasonable animal management practices will prevent cross-contamination of animals,
 - C. Pigs can become infected by virus when exposed to lawn surfaces seeded with virus.
2. Results of this study suggest that contact with lawns receiving a one-time virus application of as many as 10^4 CCID₅₀ virus per unit area may pose no significant risk of infection.

CHAPTER 8

RECOMMENDATIONS FOR FUTURE RESEARCH

Use of pigs and their associated enteroviruses has been shown to be a reasonable method for evaluating the potential risk of viral infection due to contact with lawn irrigated with treated wastewater. Preliminary research has given rise to a number of questions which could be addressed using this model. Many aspects of the use of treated wastewater for irrigation and the potential for viral infection need to be investigated in more detail. Based on the knowledge gained from the development and evaluation of this animal model, the following recommendations for future research are suggested:

1. Further assessment of conditions which may affect model system results needs to be carried out. Careful evaluation and control of confounding factors, such as differences in incidence rate of infection due to such things as litter variation, should be addressed in order to increase confidence in dose-response estimates.
2. The concentration of virus which may be applied to lawn surfaces in wastewater without significant threat of viral disease needs to be clearly established. In order to address this question experiments need to be designed which will clearly define the dose-response relationship, particularly with regard to the critical aspect of incidence rate between 10^4 and 10^5 CCID₅₀ virus exposures.

3. Repeated exposure to low concentration of virus applied to lawns may pose a risk which is significantly different from a one-time exposure. Because this situation may realistically be encountered, it should be examined in detail.
4. An important potential feature of the model is the ability to control various physical conditions through careful design of experiments. Using a dose which will give an infection incidence rate of 100, experiments should be designed to address important questions relating to wastewater management practices. These might include the effect on incidence rate of:
 - a. Time of day of wastewater application,
 - b. Environmental conditions, including: temperature, relative humidity, sunlight and soil moisture, and
 - c. Drying time between application of virus seeded water and placement of animal on the grass plot.

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VITA

Elizabeth Jane Deming was born in Pullman, Washington on July 2, 1955. She attended elementary schools in the Pullman School District and graduated from Pullman High School in 1973. Elizabeth attended the University of Southern California, and Washington State University and received the degree of Bachelor of Science in Medical Technology from California State University at Los Angeles in 1978. After working as a Radiation Protection Technologist at the University of Utah, Elizabeth enrolled at Colorado State University in 1982 to pursue a Master of Science degree in Radiation Ecology. The degree was awarded in May, 1984. The opportunity to work at Oak Ridge Associated Universities brought Elizabeth to Tennessee. In September, 1987 she entered the University of Tennessee and in December, 1991 received the Doctor of Philosophy Degree in Ecology.

While working towards her doctorate at the University of Tennessee, Elizabeth met Gary F. Caldwell in the Virology Laboratory at the University of Tennessee Veterinary Teaching Hospital. They were married in May, 1991.