Assessment of rabies antibody activity in serum of normal raccoons

by

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A Thesis Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

MASTER OF SCIENCE

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

Iowa State University
Ames, Iowa

1990

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EXPLANATION OF THESIS FORMAT

This is an alternate format thesis. The thesis begins with a general introduction and literature review followed by two separate manuscripts. A general summary and discussion concludes the thesis. The master's candidate, Richard Hill, is the senior author and principal investigator of the study.

GENERAL INTRODUCTION

Rabies virus is capable of infecting all warm blooded animals and with the exception of a few countries, is found worldwide. Today, sylvatic rabies is present throughout the continental United States. As domestic animal control programs eliminated canine rabies, wildlife host ranges expanded and increased numbers of wildlife cases were reported. Laboratory confirmed cases of rabies are most common in the skunk, raccoon, fox, and bat (Smith, 1989). In the last 30 years, the raccoon has emerged as a new reservoir host. Two epizootics of raccoon rabies have occurred in the southeastern and mid-Atlantic areas of the United States. Since 1982, the raccoon has been the second most commonly reported rabid wildlife species (Baer et al., 1990).

Rabies in raccoons is not currently a problem in Iowa, and is only sporadically reported in the central United States. Serologic investigations have shown that the prevalence of serum neutralizing antibodies in raccoons in areas without enzootic raccoon rabies is higher than the prevalence of the disease as reported by public health laboratories (McLean, 1975; Niemeyer, 1973). The role of the raccoon in the maintenance and transmission of rabies in areas without enzootic raccoon rabies is only partially understood. Raccoon susceptibility to the skunk virus has not been reported.

The purpose of this study was to investigate the prevalence and nature of serum neutralizing antibodies in raccoons in two counties in Iowa and investigate the behavior of a skunk rabies isolate in this species.

LITERATURE REVIEW

History

The origin of the term rabies is from Latin *rabere* - to rave (Beran, 1981). Rabere is derived from an old Sanskrit term *rabhas* - to do violence (Steele, 1975). Beran and Wilkinson report that the first recorded description of rabies comes from the Sumerian law code from the city of Eshnunna in approximately 2000 BC. References are made to penalties dog owners must pay if dogs known to be mad, bite persons after authorities had warned the owner of the risks of not caging their animals (Beran, 1981; Wilkinson, 1988). This indicates an early recognition of the transmissibility and severity of the disease and the public health benefits associated with attempting to limit the spread of the disease.

Other early descriptions of canine rabies are found in the writings of Democritus and Hippocrates in the 5th century BC (Smithcors, 1958). Aristotle also described canine rabies about 340 BC, but incorrectly assumed it could not be transmitted to people (Smithcors, 1958). The Roman, Cardanus, was the first to recognize the infectivity of the saliva and described the infectious agent as a poison (Steele, 1975; Wiktor, 1985). There are numerous other accounts of rabies in ancient times (Beran, 1981; Smithcors, 1958; Steele, 1975; Wilkinson, 1988), most of which refer to the dangers of dog bites and the recognition that the disease is spread via the bite wound. In approximately 100 AD, Celsus made references to rabies in wild and domestic animals and people (Steele, 1975; Wilkinson, 1988; Wiktor, 1985). Being a physician, he recommended cupping or

sucking the wound followed by heat cautery, caustics, salt, or blood letting as treatments. Soranus, a Greek physician, also reported the possibility of wild animals transmitting the disease (Steele, 1975).

In the centuries that followed, many preventive and post-exposure treatments were proposed (Beran, 1981; Steele, 1975; Wilkinson, 1988). Such references demonstrate that the disease was well understood, but considering the medical skills of the times, little could be done excepting local treatment of the wound. Assorted treatments included hot and cold water baths, oil treatments to the wound, application of goose grease or honey to the wound, consumption of large quantities of wine, eating cock's brains or the salted flesh of the rabid dog, and countless other assorted superstitious treatments (Steele, 1975).

Reports of world wide epidemics of canine rabies from the 1500s to the 1800s are well documented (Beran, 1981; Smithcors, 1958; Steele, 1975). In the United States, early reports of canine rabies came from Virginia in 1753, North Carolina in 1762 (Beran, 1981; Smithcors, 1957), Boston in 1768, and Pennsylvania and Maryland by 1799 (Smithcors, 1957). In 1779, James Mease, a Pennsylvania physician published a work: On the Disease Produced by the Bite of a Mad Dog, insisting the only way to spread the disease was by a bite wound (Smithcors, 1957). Following the Civil War, canine rabies was widespread in most of the United States (Beran, 1981; Smithcors, 1958; Tierkel, 1975).

Similarly, world wide outbreaks of wildlife rabies were being reported. Wolf rabies was recognized as early as the 12th century in France and the 13th century in Germany and Asia (Beran, 1981; Smithcors, 1958). As early as 1576, Spanish

explorers reported bat rabies in Central and South America, and death in cattle following the bites of bats (Beran, 1981; Baer, 1975b). In North America, early Eskimo folklore describes a disease in foxes fitting the description of rabies which could be transmitted to dogs and people (Beran, 1981). By the 1700s rabies was enzootic in foxes in the eastern United States (Winkler, 1975). Rabies was recognized in several other terrestrial wildlife species across the United States by the 1800s (Beran, 1981; Parker, 1975; Winkler, 1975).

Attempts to control epidemics of rabies were aimed at the dog and included population reductions, animal control, and muzzling orders imposed and regulated by governmental authorities (Beran, 1981; Steele, 1975). With few exceptions, most of these attempts were unsuccessful (Steele, 1975). One exception was the successful rabies eradication in Great Britain by 1902 using these methods (Steele, 1975).

It was not until the late 1800s with the research of Pasteur and his co-workers that dramatic changes occurred in the world wide picture of rabies. With the exception of animal avoidance and the treating of bite wounds, little changed until the advent of the first effective post-exposure prophylactic vaccine by Pasteur in 1885 (Beran, 1981; Steele, 1975; Wiktor, 1985). Beginning in 1881 with the experimental transfer of infection by nervous tissue, Pasteur, without knowledge of the causative agent, created an attenuated vaccine using dried pieces of spinal cord from rabbits infected with virus with a fixed incubation period of seven days (Steele, 1975; Wiktor, 1985). Pasteur's immunization protocol consisted of injecting preparations of spinal cord which had been dried

for 14 and subsequently fewer days (Beran, 1981; Steele, 1975).

In the last 105 years since the development of a successful human vaccine, there have been numerous accomplishments in the areas of rabies control. One of the first vaccines to be widely used in domestic animals was a vaccine produced from fresh rabbit spinal cords in 1898 by Hogyes (Bunn, 1988). In 1908, Fermi developed a phenolized vaccine which was more stable than the spinal cord preparations of Pasteur (Steele, 1975). Semple continued the work on an inactivated vaccine and produced a highly antigenic phenolized vaccine in 1911 (Beran, 1981). In 1955, Fuenzalida and Palacios reported an inactivated suckling mouse brain vaccine which decreased the demyelinating neuroparalytic hazards of other vaccines made from nervous tissue origin (Beran, 1981; Kaeberle, 1958; Wiktor, 1985). Since then, many different types of rabies vaccine have been prepared from nerve tissue of animal origin. In addition to using these vaccines for human beings, mass dog vaccination programs as a means of rabies control were started as early as 1919 (Bunn, 1988).

Modified live virus vaccines of avian embryo origin were developed in 1948 (Koprowski and Cox, 1948). Steele reports that these vaccines resulted from early successes of Dawson in 1930 and Bernkopf and Kligler in 1940 in growing rabies virus in developing embryos (Steele, 1975). Mass application of these potent vaccines in the late 1940s and early 1950s led to a significant decrease in canine rabies in many parts of the world (Baer 1990). However, cases of vaccine induced rabies were seen with some of these avian embryo origin vaccines (Pederson et al., 1978; Whetstone et al., 1984).

Steel reports that in 1936 there were two major developments in rabies virus research; the reporting of the size of the virus by Galloway and Elford and the first propagation of rabies virus in tissue culture by Webster and Clow (Steele, 1975). In 1958, the rabies virus was adapted to cell culture (Kissling, 1958). With this new technology, extensive work on many tissue culture origin vaccines was done, leading to the many different types of vaccines that exist today (Beran 1981; Bunn 1988). These newly developed vaccines had the advantage of reducing excessive anaphylactic reactions caused by tissue antigens in avian embryo origin vaccines (Wiktor, 1985). Most of the vaccines available for domestic animals in the United States today are killed virus cell culture origin products (Bunn, 1988).

The major emphasis in vaccine production has been for use in domestic animals rather that in wildlife. Baer reports that as early as 1960, work was started on the vaccination of wildlife species (Baer,1988a). Effective oral vaccines for wildlife have been used in numerous countries throughout the world (Steck et al., 1982a and 1982b; Rupprecht et al., 1988).

In addition to vaccination and control programs, the diagnosis of rabies has historically presented many difficulties for the medical profession (Steele, 1975). The finding of pathognomonic Negri bodies by Negri in 1903 was a key event in diagnosis (Baer et al., 1990). Although he had incorrectly identified these inclusion bodies as protozoa, he had identified the horn of Ammon as the site of predilection (Steele, 1975). Later in 1927, Sellers demonstrated that Negri bodies could be identified in impression preparations of brain tissue (Steele, 1975).

The next advance in rabies diagnosis was the mouse inoculation test (Webster and Dawson, 1935). This test has become the accepted standard and is widely used today. A similar test capable of appraising the potency of vaccines was developed by Habel in 1940 (Beran, 1981; Bunn, 1988). The fluorescent antibody (FA) test also had a significant impact on rabies diagnostic procedures. Goldwasser and Kissling reported the development of a FA test for the diagnosis of rabies in central nervous system tissue in 1958 (Goldwasser and Kissling, 1958). Several other diagnostic tests have been adapted to aid in the diagnosis of rabies in other tissues, and numerous serologic tests have been developed (Webster and Casey, 1988). These and other recent accomplishments are discussed separately in following sections.

Etiology

Rabies viruses are members of the family Rhabdoviridae and are considered the type species of the genus Lyssavirus (Baer et al., 1990; Wagner, 1990). They are believed to have originated in Africa (Beran, 1981). Rhabdoviruses are enveloped bullet shaped RNA virions that measure an average of 75 by 180 nm (Wunner et al., 1988). They are found worldwide and afflict over 100 species including plants, reptiles, fish, crustaceans and mammals (Baer et al., 1990). In addition to rabies virus, five other viruses in the genus Lyssaviruses are commonly referred to as the rabies-related organisms. These include Duvenhage, Kotonkan, Lagos Bat, Mokola and Obodhiang viruses (Beran, 1981; Kaplan, 1985; Shope, 1975; King and Crick, 1988). Monoclonal antibody studies have allowed

classification of the Lyssaviruses into four distinct serotypes (World Health Organization, 1984). Serotype 1 includes the prototype strain Challenge Virus Standard (CVS) and the majority of laboratory and field strains. Serotypes 2, 3, and 4 are the rabies-related viruses. Other viruses such as the Nigerian horse virus, Ouluo-Fato, Bolivar and Rodent virus have also been reported to have morphological and serological relatedness to rabies virus (Kaplan, 1985; Nawathe and Lamorde, 1982). Monoclonal antibodies directed against nucleocapsid and glycoprotein antigens have allowed further classification of different virus strains (Smith et al., 1986).

Virus Structure, Function and Characteristics

The virus consists of nucleic acid and five proteins (G, N, M1, M2 and L) (World Health Organization, 1984). The virions are bound in a lipoprotein (lipid and M2 protein) envelope which has glycoprotein G transmembrane spikes (Greene et al., 1984, Crick and King, 1988, Tordo and Poch, 1988). The 69 kilodalton (kDa) G-protein is the only polypeptide that is glycosolated and completely spans the envelope (Wunner, 1985). There are no spikes on the planar end of the virion (Wunner, 1985).

Glycoprotein G is responsible for induction and binding of virus neutralizing antibodies and protection of animals against challenge. In addition, the glycoprotein is responsible for mediating attachment of the virus to host cells, determining virulence and stimulating T cells which express suppressor, helper, or cytotoxic activities (Baer et al., 1990; Crick, 1985; Dietzschold et al., 1985). The

nucleotide sequence of the G protein has been determined for several strains (Tordo and Poch, 1988; Dietzschold et al., 1985). Monoclonal antibodies have been used to construct operational antigenic maps of the rabies glycoproteins (Flamand et al., 1980b; Lafon et al., 1984). Three functionally independent antigenic sites in CVS-11 and five distinct sites in the Street Alabama Dufferin (SAD) strain have been defined (Wunner et al., 1988). There is also evidence of new antigenic sites on both the CVS and SAD strains (Bunschoten et al., 1989).

The core of the virus particle is a tightly structured ribonucleoprotein helix consisting of the RNA genome and the internal proteins (Wunner et al., 1988). The genome is unsegmented single stranded non-infectious RNA of negative polarity with a molecular weight estimated at 3.5 to 4.6 x 10^6 Daltons (Crick and King, 1988; Tordo and Poch, 1988). The complete sequence of 11932 nucleotides has been determined (Tordo and Poch, 1988).

Within the ribonucleoprotein complex are approximately 1,800 copies of the 55 kDa phosphorylated N protein core in association with approximately 900 copies of the 400 kDa M1 protein and 30-60 copies of 244 kDa L protein (Baer et al., 1990; Crick and King, 1988; Dietzschold et al., 1985). A matrix M2 protein on the inner side of the lipid envelope interacts with both the lipid bilayer and the ribonucleoprotein core (Tordo and Poch, 1988). The N protein contains the cross reactive group specific antigen of the rabies group of viruses and is responsible for shared complement fixing, immunoprecipitation and immunofluorescence tests of these viruses (Beran, 1981; Baer et al., 1990; Dietzschold et al., 1985). The N protein also plays a role in the induction of

protective antibody titer (Dietzschold et al., 1987). The M-proteins are thought to play a role in the budding process (Baer et al., 1990). Monoclonal antibody studies have identified distinct antigenic sites in the nucleoprotein (Flamand et al., 1980a).

Chemical analysis reveals that purified virus contains 3-4% RNA, 67% protein, 3% carbohydrate associated with proteins and 26% lipid (Schneider and Diringer, 1976; Sokol, 1975). The virus is inactivated by lipid solvents, 45-70% ethanol, iodine preparations and quaternary ammonium compounds (Kaplan, 1973). The virus is unstable at pH less than 5 or greater than 10 (Clark and Wiktor, 1972). Desiccation, sunlight, ultraviolet and x-irradiation, trypsin, ether and beta-propiolactone readily inactivate the nucleic acid (Kaplan, 1973; Martin and Sedmak, 1983). The virus is stable for many years when frozen at -60 to -80° F (Bernard and Fishbein, 1990). The virus is also stable in carrion. The virus survives in animal tissue for 20 days (10° C) and 8 days (25° C) (Soave, 1966).

Clinical Signs

Rabies virus is capable of infecting all warm blooded animals, but species susceptibility varies considerably (World Health Organization, 1973). Opossums appear least susceptible; moderately susceptible are dogs, human beings and most wildlife reservoirs. Foxes, coyotes, jackals and wolves appear most susceptible (Baer et al., 1990; World Health Organization, 1984). The rate of spread differs with species and with age (Martin and Sedmak, 1983). Incubation periods are

highly variable and are dependent upon many factors such as site of bite, innervation at bite site, amount of virus injected, species bitten, immune status, species adapted variant of virus and age of host (Acha and Szyfrez, 1987; Baer et al., 1990; Charlton, 1988; Greene et al., 1984). Examples of the variability of these factors have been demonstrated; incubation periods are shorter in bites to the head (Baer et al., 1990), and young dogs are more susceptible than adults (Tierkel, 1975).

On a worldwide basis, dogs are the major reservoir of rabies in animals (Beran, 1981). They have been considered the prototype species for the pathogenesis of the disease (Tierkel, 1975). Clinical signs are divided into three major stages; prodromal, excitement and paralytic. The incubation period is usually three to eight weeks, but periods as short as 11 days, or as long as six months are documented (Beran, 1981; Fekadu et al., 1982). The prodromal phase generally lasts for two days with behavior changes, apprehension and temperament changes most commonly reported (Beran, 1981; Greene et al., 1984). The excitement phase usually lasts for one to seven days, but may be so transient that it is unrecognizable (Greene et al., 1984). Clinical signs include restlessness, irritability, increased responses to auditory and visual stimuli, photophobia and snapping at imaginary objects. As dogs become more restless, they become more irritable and vicious (Beran, 1981; Greene et al., 1984). The paralytic phase usually lasts from one to four days. Progressive paralysis starts in the area of exposure, posterior extremities, or the lower jaw (Beran, 1981; Tierkel 1975). Cranial nerve paralysis is seen where bites have occurred around the face

(Baer et al., 1990). Salivation, excessive frothing and dropped jaw may occur with paralysis of the masticatory and laryngeal muscles (Tierkel, 1975). Paralysis spreads rapidly to the rest of the body, and death follows as a result of coma and paralysis of respiratory muscles. Rare abortive forms of the disease have been documented (Fekadu and Baer, 1980).

The phases of disease in other animals are comparable to the dog with variability among species (Beran, 1981; Greene et al., 1984; Kaplan, 1985). Notable changes in other species include the excitement phase seen in a majority of cats and unusual behavior changes commonly associated with wildlife species (Beran, 1981; Greene et al., 1984). Many wild animals lose their fear of people, and nocturnal animals come out in daylight (Beran, 1981; McLean, 1975).

Pathogenesis

Several distinct steps are involved in the pathogenesis of rabies as the virus moves through the body (Charlton, 1988). These include introduction of the virus via a bite wound, laceration and less commonly mucus membranes; migration via peripheral nerves to the central nervous system (CNS); and centrifugal neural transport and infection of non-nervous tissue (Charlton, 1988). Following inoculation into a muscle field, the virus can only be recovered for a limited period of time, usually less than 24 hours (Baer, 1975a; Charlton, 1988; Tsiang, 1988). The virus becomes sequestered followed by replication in monocytes (Baer et al., 1990; Beran, 1981; Murphy, 1985). Virus penetration into host cells is either by adsorptive endocytosis or by direct fusion with cell membranes

(Tsiang, 1988). During this initial period, there is an eclipse phase where neither antigen nor virus can be identified (Baer et al., 1990). Commonly, there is no viremia unless it follows a high inoculum dose in experimental animals (Murphy, 1985).

Next, the virus traverses neuromuscular and neurotendinous spindles to approach peripheral nerves (Murphy, 1985). The mechanisms of neuromuscular virus passage are not clear. Acetylcholine receptors on the virus have been suggested as one method of facilitating cellular uptake (Baer et al., 1990; Charlton, 1988). Other researchers have concluded that rabies virus interaction with cell surfaces is via carbohydrate moieties, phospholipid and highly sialyated gangliosides which are independent of acetylcholine (Tsiang, 1988).

The bite route is by far the most common method of transmission, but other routes are documented. Viral infection of olfactory epithelial cells and bipolar neurons extending to the olfactory bulb has been reported (Charlton, 1988). Aerosol transmission is reported in both humans (Winkler et al., 1973) and animals (Winkler et al., 1972). Viral progression to the central nervous system from oral mucus membranes is believed to be via the neuroepithelium of taste buds (Baer, 1975a). Infection via the cornea is probably through uptake of sensory nerve fibers and not the optic nerve (Charlton, 1988). Other unusual forms of pathogenesis include viral entry via the gastrointestinal route (Ramsden and Johnston, 1975), sequestration of virus in brown fat of bats (Martin and Sedmak, 1983), and lactogenic or transplacental transmission (Howard, 1984).

Once the virus enters nerves, it is believed to progress in the tissue spaces

between the Schwann cells and perineural structures (Martin and Sedmak, 1983). It is reported that fixed rabies virus travels at approximately 3 mm per hour (Dean et al., 1963). Rabies viral transport to the CNS can occur along motor or sensory fibers (Charlton, 1988). Blood-vascular routes have also been reported as means of transfer to the CNS (Baer, 1975b).

Viral replication occurs in the dorsal root ganglia or spinal ganglia corresponding to the involved peripheral nerves and then progresses rapidly to the CNS (Baer, 1975a). The virus may be hidden from the immune and inflammatory systems of the host (Murphy, 1985). Selective areas of replication of the virus occur in neurons, and spread of infection in the CNS is considered trans-neuronal via formation of virions on cell membranes (Charlton, 1988). There is tropism for the neuronal cells of the brainstem, hippocampus, subcortical nuclei, limbic cortex and Purkinje cells in the cerebellum (Charlton, 1988; Martin and Sedmak, 1983; Schneider, 1975a).

Once the virus reaches the brain, peripheral nerves act as pathways for rapid centrifugal spread of virus throughout the entire body (Baer, 1975a; Charlton, 1988). This accounts for occurrences of virus in tissues and fluids before the onset of clinical signs (Beran, 1981; Charlton, 1988). Of all non-neural tissues, the salivary glands are most likely to contain the rabies virus (Schneider, 1975b). The glands are infected via release and entry of the rabies virus into acinal epithelial cells from the terminal axons (Charlton et al., 1983). Virus budding from plasma membranes of mucous acinar cells delivers virus into the secretions (Martin and Sedmak, 1983). Rabies virus has been isolated in the saliva of dogs

for up to 10 months following experimental infection with a challenge prepared from saliva from Ethiopian dogs (Fekadu et al., 1981; Fekadu and Baer, 1980). Virus has also been demonstrated in numerous other tissues and fluids (Martin and Sedmak, 1983).

The majority of rabies infections progress through typical pathogenesis of the disease characterized by the spread of the virus, development of classical clinical signs and death. Although uncommon, numerous variations exist in the pathogenesis (Charlton, 1988). Experimentally observed variations include recovery from infection with or without disability (Fekadu and Baer, 1980), variable or prolonged incubation period (Fekadu et al., 1982), varying periods of clinical signs (Perl et al., 1977; Schneider 1975b), change in type of clinical signs (Charlton, 1988), variations in virus excretion (Schneider, 1975b), and development of the carrier state (Martin and Sedmak, 1983; Perl et al., 1977). Although definitive explanations for these variations have not been found, possibilities include route of exposure and dose of inoculum (Charlton, 1988), species adapted virus variants (Smith et al., 1986), genetic resistance (Lodmell, 1988), and immunologic mechanisms (MacFarlan, 1988).

Pathology

A limited number of lesions are observed when considering the severity of the symptoms seen in rabies. Congestion of meningeal vessels is commonly the only gross lesion seen (Perl, 1975). Pathologic changes include encephalomyelitis characterized by diffuse and perivascular cuffing, neuronophagia, neural

degeneration and neuronal intracytoplasmic inclusion bodies (Negri bodies) (Atanasiu, 1975; Perl, 1975). Negri bodies are ribonucleoprotein, they appear as acidophilic inclusions with basophilic granules, and are most abundant in the central pyramidal layer of the hippocampus (Atanasiu, 1975). Special stains such as Sellers stain have been developed to help elucidate them (Tierkel, 1973). Pathologic changes vary with the stage of infection, part of the CNS affected, immune and inflammatory response, type of virus variant causing the disease and species affected (Atanasui, 1975; Baer et al., 1990; Lepine, 1973b; Perl, 1975; Tierkel, 1973).

Diagnosis

Diagnosis of rabies based on epidemiological studies or clinical signs is very important, but only through laboratory tests can rabies be confirmed (Baer et al., 1990). Tests for rabies antigens or antibodies are the primary tests because routine chemical and hematological tests do not usually show specific abnormalities (Hattwick and Gregg, 1975).

In human beings, many non-invasive antemortem tests are available (Baer et al., 1990), including 1) electroencephalography (EEG) which may be normal early in illness yet may show abnormalities as the disease progresses, 2) CNS imaging which may be normal or show CNS edema and increased intracranial pressure, 3) Examination of full thickness skin biopsy from the nape of the neck by fluorescent antibody technique, 4) virus isolation from saliva by mouse inoculation or other antigen tests, 5) FA testing of corneal impression smears, 6) detecting of

neutralizing antibodies in serum or cerebral spinal fluid. Many of these same tests can be performed in animals, but some are impractical.

The most common post mortem diagnostic tests are antigen detection tests. One of the standard tests is an animal (primarily mouse) inoculation test with suspect brain tissue followed by FA staining (Koprowski, 1973). This test is highly specific, but is time consuming and successful only on fresh or frozen tissues (Atanasui, 1975; Greene et al., 1984; Koprowski, 1973). Observation of stained Negri bodies in fresh or formalized tissues is also highly specific, but may be restricted by appearance of the Negri bodies late in the course of disease. Also, highly virulent strains may not produce many inclusion bodies (Atanasui, 1975; Tierkel, 1973).

The most widely used method of antigen detection is the direct immunofluorescence test (Greene et al., 1984). The test is rapid and specific in the hands of experienced personnel (Beran and Crowley, 1983; Dean and Abelseth, 1973). In many laboratories, sensitivity of the results is increased by performing the mouse inoculation test in all FA negative samples (Dean and Abelseth, 1973). Immunoperoxidase and FA tests have also been adapted to an ante mortem test to detect antigen in corneal impression smears, tactile hair roots, or skin biopsies (Blenden, 1981; Blenden et al., 1983; Ciuchini et al., 1984).

Other methods for antigen detection include cell culture inoculation followed by FA staining (Wiktor, 1973b), or plaque assay (Crick and King, 1988), enzyme immunoassay on both fresh and fixed tissues (Bourgon and Charlton, 1987; Webster and Casey, 1988), complement fixation (Kuwert, 1973a),

hemagglutination (Kuwert, 1973b), gel immunoprecipitation (Lepine, 1973a), and electron microscopy (Hummeler and Atanasiu, 1973). Non-infections virion free antigen produced in cell culture can be detected by complement fixation and gel diffusion tests (Wiktor, 1973b). Monoclonal antibodies directed against the glycoprotein and the ribonucleoprotein of matrix proteins are available to help identify rabies and rabies-related virus isolates (Dietzschold et al., 1990; Wiktor and Koprowski, 1980).

Where post-mortem samples are available, these tests provide confirmation of suspected clinical rabies and allow epidemiologic studies on the nature of the virus. Monoclonal antibodies directed against nucleocapsid proteins of rabies virus isolates have been the most helpful in epidemiologic diagnosis and analysis of outbreaks (Smith, 1989).

A wide variety of serological antibody detection techniques are available. The virus neutralization test in mice is the accepted standard of comparison for all other antibody tests (Atanasiu, 1973). Fluorescent antibody techniques, especially the Rapid Fluorescent Focus Inhibition Test (RFFIT) (Smith et al., 1973), are the most widely accepted alternatives to the mouse neutralization test (Campbell and Barton, 1988; Martin and Sedmak, 1983). These are very easy to perform and provide rapid results. Modifications of these tests allow the measurement of specific immunoglobulins (Campbell and Barton, 1988). Enzyme-linked immunosorbent assays which correlate well with the mouse test are available (Campbell and Barton, 1988; Grassi et al., 1989).

Other methods for the detection of rabies antibodies include a plaque

neutralization test (Wiktor, 1973b), complement fixation (Kuwert, 1973a), hemagglutination inhibition (Kuwert, 1973b), passive hemagglutination (Dierks and Gough, 1973), gel immunoprecipitation (Lepine, 1973a), radioimmune assay (Wiktor, 1973a), indirect immunofluorescence (Johnson and Emmons, 1980), soluble antigen fluorescent antibody test (Garnam et al., 1977), and dot immunobinding (Heberling et al., 1987). Serologic tests are used primarily for assaying immune status in animals and people following vaccination, or for epidemiologic studies. Varying immune responses to natural infection limit the interpretation in potentially infected animals (Murphy, 1977).

Epidemiology

In spite of improved technology, rabies continues to spread in the world (Beran and Crowley, 1983). In 1988, with the exception of 60 countries, rabies was found worldwide (Baer et al., 1990). Throughout the world, rabies exists and is maintained as an infection in different principal hosts which also may act as the reservoir species (Beran, 1981). Two distinct epidemiological forms characterized as urban and sylvatic cycles are recognized (Steele, 1988).

In the urban cycle, the dog is the principal vector for transmission to other animals (Acha and Arambulo, 1985, Beran, 1981). This is especially true in developing countries. Wild animals may be infected by dogs and then spread the disease to people, but they play only a minor role in the rabies cycle (Beran and Crowley, 1983). In most countries of the world, the incidence of human rabies closely parallels the incidence of disease in dogs. Almost all cases of human

rabies originate in countries with uncontrolled canine rabies (Baer et al., 1990). The true number of human rabies cases worldwide can only be estimated. Acha reported 20,482 confirmed human deaths in 1981 and over 500,000 persons receiving post-exposure rabies treatment (Acha and Arambulo, 1985). In India, China and Thailand, it is estimated that 45,200 to 57,300 people die of rabies each year and over 3 million people receive post-exposure treatment (Baer, 1988b). It is widely recognized that the number of rabies cases is grossly under reported (Baer, 1988b; Warrell and Warrell, 1988).

Sylvatic rabies consists of two distinct groupings; terrestrial animal rabies and bat rabies. Bat rabies does not have geographically distinct outbreak areas and bat rabies exists as enzootics independent of the cycle in terrestrial animals (Smith, 1989). Wildlife species of the canidae family are the principle terrestrial reservoirs and distinct outbreak areas exist (Acha and Arambulo, 1985). Avian species can be experimentally infected with rabies, but birds are not highly susceptible and are of limited importance in the epidemiology of rabies (Shannon et al., 1988). The predominant reservoir species worldwide are as follows: (Acha and Arambulo. 1985; Everard and Everard, 1988; Kaplan, 1985; Jenkins et al., 1988, Hubschle, 1988; Smith 1989; Pacer et al., 1985)

Africa
- kudo antelopes (*Tragelaphus strepsiceros*)
- jackals (*Canis mesomelas*)
- wolves (*Canis lupus*)
- mongooses (*Viverridae*)
- dogs (*Canis familiaris*)

Asia
- artic foxes (*Alopex lagopus*)
- wolves (*Canis lupus*)
- dogs (*Canis familiaris*)

Central America	- mongooses (*Viverridae*) - dogs (*Canis familiaris*)
Europe	- red foxes (*Vulpes vulpes*) - raccoon dogs (*Nyctereutes procyonoides*)
North America	- skunks, primarily striped skunk (*Mephitis mephitis*) and spotted skunk (*Spilogali putorius*) - raccoons (*Procyon lotor*) - red foxes (*Vulpes vulpes*) - artic foxes (*Alopex lagopus*) - grey foxes (*Urocyon cineroargenteus*)
South America	- vampire bats, primarily common vampire (*Desmodus rotundus*) - dogs (*Canis familiaris*)

Various mechanisms are responsible for the maintenance and spread of rabies virus in nature (Martin and Sedmak, 1983). Epidemiologically the most important route of transmission is by bite wound and transfer of saliva. Although all warm blooded animals are able to become infected and spread the disease, the adaptability of a strain to a particular host is very important. Maintenance and spread depend largely on the ecology of the host species (Kaplan, 1985). Other factors affecting transmission are the variable incubation period of the virus and variability of secretion of virus in saliva. Uncommon occurrences not completely understood include subclinical infections with shedding, latent infections, and recovered healthy carriers (Baer, 1975a; Beran, 1981; Kaplan, 1985; Martin and Sedmak, 1983). All of these assure maintenance of infection despite the usually fatal course of the disease.

Rabies in the United States

Rabies in the United States has changed dramatically in the last 40 years (Beran, 1981; Pacer et al., 1985). With the advent and widespread use of effective domestic animal vaccines in the 1950s, dog rabies decreased dramatically from 8000 to 10,000 cases per year to less than 100 per year (Pacer et al., 1985). At the same time, wildlife rabies has been increasing (Baer et al., 1990; Smith, 1989). In 1988, 4724 laboratory confirmed cases were reported in the United States and its territories (Centers for Disease Control, 1989). Of these, 4174 (88.4%) were wildlife species (Centers for Disease Control, 1989) which is estimated to represent only one to ten percent of the actual incidence (Beran, 1981).

Laboratory confirmed cases in wildlife species are most common in the skunk, raccoon, fox and bat with skunks and raccoons accounting for the majority of the cases (Smith, 1989). In 1989, skunks accounted for 37.9% of the rabies in animals (Centers for Disease Control, 1989) whereas in 1953 skunk rabies was recognized in only 5 states and accounted for only 3.6% of the cases (National Communicable Disease Center, 1964). Since 1960, skunks have been the animal most frequently reported rabid in the United States (Centers for Disease Control, 1977 and 1989). An epizootic of raccoon rabies in the Eastern United States has made this animal the second most common rabid wildlife species since 1982 (Baer et al., 1990). In 1989, 31.1% of the total wildlife cases were in raccoons (Centers for Disease Control, 1989). The increase in this species has been dramatic. Raccoon rabies was only sporadically diagnosed until it was recognized

in the southeast in the late 1940s (Kappus et al., 1970), and in the mid-Atlantic states in the 1970s (Jenkins et al., 1988). In contrast, fox rabies has been decreasing. In 1953, 1033 cases were reported and only 183 cases reported in 1988 (Centers for Disease Control, 1989; National Communicable Disease Center, 1964). Bat rabies has not changed much over the same time period. In 1988, bats accounted for only 0.14% of the total rabies versus 0.091% in 1953 (National Communicable Disease Center, 1964; Centers for Disease Control, 1989).

There are five geographical areas in the United States where antigenically distinct enzootic rabies occurs and all are in association with different wildlife species (Smith 1989). Within these areas, large numbers of rabies cases are reported in one major host species with only rare occurrence in other species (Smith, 1989). Monoclonal antibody studies directed against nucleocapsid proteins of rabies virus isolates have shown an ecotype species/geographical association (Smith et al., 1986). The skunk is the predominant reservoir host in a belt in the central United States from Minnesota to Texas and in northern California (Beran, 1981; Smith and Baer, 1988). Two distinct virus variants are observed in the northern and southern areas of this region (Smith et al., 1986). Striped skunks, and with less frequency spotted skunks, are the predominate species with hooded skunks and hog nose skunks seldom reported (Parker and Wilshack, 1966; Pacer et al., 1985). Raccoon rabies is found mainly in southeastern states and in middle eastern seaboard states of Maryland, Delaware, Virginia, Pennsylvania and West Virginia (Jenkins et al., 1988). Red fox rabies is seen in northern New York, grey fox rabies in areas of Texas and Arizona

(Kaplan, 1985). Bat rabies is reported in most states (Smith et al. 1986). Human rabies cases are sporadically reported, with only 12 cases reported within the last 10 years, yet approximately 30,000 people receive post-exposure prophylactic treatment each year (Centers for Disease Control, 1989).

Rabies in Iowa

In Iowa, the skunk is the major reservoir and in 1989 accounted for 113 of 203 total laboratory confirmed animal cases (Iowa Department of Public Health, 1990). Of the other cases in 1989, 71% occurred in cattle and cats because of spill-over into these species which are highly susceptible and not commonly vaccinated (World Health Organization, 1984).

It is uncertain how skunks transmit virus among themselves and other species, but many factors are considered. Skunks occupy communal dens with single adult males in the winter; but in late winter, conflicts with wandering unattached males may allow for easy transmission and dispersal (Parker, 1975). High population density and turnover also help maintain the infection (Fernandes and Arambulo, 1985; Sargeant et al., 1982). Rabies infected skunks have been shown to travel widely (Storm and Verts, 1966). It is also common for adult males and juveniles to disperse over long distances (Hall, 1981). Highly variable incubation periods of 14 to 177 days in natural infections (Sikes, 1962), would allow for maintenance of the virus over winter denning periods (Parker, 1975). Skunks can shed large amounts of virus in their saliva and oral transmission has been reported which may allow for transmission in the dens (Parker, 1975).

Rabies in Raccoons

The natural habitat of raccoons ranges from southern Canada to Panama, including all areas of the continental United States except high mountains and deserts (Kaufmann, 1982). Raccoons have been introduced in areas of the Soviet Union and are established in several European countries (McLean, 1975; Artois et al., 1989). Raccoons are considered to be highly intelligent and able to live in close association with people in both urban and rural areas, often thriving on human garbage (Hoffman and Gottshang, 1977).

McLean reports that the first known case of rabies in raccoons was found in California in 1936 (McLean, 1975). Prior to 1950, there were only sporadic reports of rabies in raccoons across the United States (McLean, 1975). Since then, two epizootics of raccoon rabies have been recognized (Jenkins et al., 1988). The first epizootic started in Florida from a focus of one positive raccoon in 1947 (Bigler et al., 1973). By 1959, the raccoon was the most commonly reported rabies host in that state (McLean, 1975). The disease extended northward through Florida and into the surrounding states of Georgia, Alabama and South Carolina at a rate of approximately 25 miles per year (Kappus et al., 1970; McLean, 1975). Before 1962, only 2 cases of rabies had been reported in Georgia. In 1963, the number of cases of raccoon rabies in the United States increased to over 100 for the first time and the raccoon was identified as an emerging wildlife host (National Communicable Disease Center, 1964). Today all of Florida, most of Georgia and South Carolina and eastern Alabama are enzootic for raccoon rabies (Pacer et al., 1985).

In 1977, a similar epizootic occurred in the middle Atlantic states which has now extended into Delaware, Maryland, Pennsylvania, Virginia, West Virginia and the District of Columbia (Hubbard, 1985). Cases of rabies went from sporadic reports and one positive case in 1977 to 1608 cases in 1983 (Jenkins et al., 1988). The highest number of cases of rabies in raccoons in the United States occurred in 1983 when 1820 cases were reported (Centers for Disease Control, 1984).

It is unknown why these regional epizootics occurred. In Florida, habitat destruction and decreased rainfall have been suggested as causative factors (Hubbard, 1985). Also, physiological stress due to overcrowding has been suggested (McLean, 1975). The mechanism for the mid-Atlantic outbreak is probably due to translocation of Florida raccoons to the area for restocking purposes (Smith et al., 1984). Monoclonal antibody studies have shown similar antigenic characteristics in rabies virus isolates from the two areas (Smith et al., 1984).

Control

Throughout the world, rabies is maintained in enzootic areas because of the presence of reservoir animals (Beran and Crowley, 1983). From these animals, the disease is spread to other susceptible hosts including people (Kaplan, 1985; Martin and Sedmak, 1983). Effective control measures involve breaking the cycle of transmission between susceptible hosts (Steele, 1988). The strategy of breaking the cycle depends on many factors some of which include the reservoir population

distribution, density and ecology of the reservoir host, and providing protection to alternate hosts from exposure or infection (Beran, 1981; Kaplan, 1985). If we consider the goal of animal rabies control programs to be reduction of rabies in people, there are distinct areas where the transmission cycle can be broken. These include prevention or elimination of disease in animals, avoidance of exposure to rabid animals, pre-exposure and post-exposure prophylaxis including wound care, and active and passive immunization (Baer et al., 1990). Control programs aimed at human vaccination are not epidemiologically sound and are not the most economical way to control rabies. Therefore, control methods must be aimed at reducing animal rabies.

Control programs in areas where dog rabies is dominant have included such methods as population reduction, restriction on animal movements, and mass vaccination programs (Beran and Frith, 1988). Dog control, vaccination programs and the removal of unrestricted animals offer the best method of canine rabies control (Beran and Frith, 1988; Middaugh and Ritter, 1982).

Population reduction methods and mass vaccination programs have been the most widely used control programs for terrestrial wildlife species (Kaplan, 1985; Lewis, 1975). The aim of population reduction is to lower the reservoir host animal density to a point below the minimum threshold to allow rabies to maintain infection without eradicating the population (Baer, 1985; Kaplan, 1985; Sedmak and Martin, 1984). Methods that have proved successful include shooting, den gassing, trapping and poisoning (Lewis, 1975,; Rosatte et al., 1986). However, as with dog rabies control programs, population reduction methods

have had only limited success and do not offer permanent solutions due to animal migration (Baer, 1988b; Wilhelm and Schneider, 1990). Humane interests are also a factor in population reduction programs.

Immunization programs aimed at wildlife were used as an alternate method of wildlife rabies control as early as 1962 (Baer, 1988b). Although parenteral vaccination of wildlife is successful in the laboratory (Blancou et al., 1986), and in the field (Rosatte et al., 1990; Thiriart et al., 1985), widespread parenteral vaccination of wildlife is not practical and there is no licensed rabies vaccine available for such use in wild animals in the United States. The first successful oral vaccination of wild animals in the laboratory occurred in foxes in 1971 with an attenuated vaccine (Baer et al., 1971). The first field trial using an oral vaccine was carried out in Switzerland in 1978 where the spread of the disease in foxes was controlled (Steck et al., 1982a, 1982b, Wandeler, 1988). Recent success in several countries with the use of attenuated modified live vaccines makes oral vaccination of wild animals and widespread control of wildlife rabies more feasible (Crick, 1985; Wandeler, 1988). Advances in genetically engineered vaccines also provide mechanisms for vaccinating wildlife. A live raccoon poxvirus vectored vaccine has proven successful in a number of species (Esposito, 1989). A live vaccinia virus vectored vaccine with the raccoon and fox as the primary target species has been found to be effective in laboratory experiments in numerous species (Rupprecht et al., 1988). This vaccine has been used in field trials in Europe (Blancou et al., 1989; Wiktor et al., 1984), and the United States (Associated Press, 1990). A live adenovirus vectored vaccine is also reported

(Prevec et al., 1990).

In addition to successful vaccination of the target species with these genetically engineered vaccines, vaccinated animals can be distinguished from naturally exposed animals because only the rabies virus glycoprotein gene has been inserted into the vector (Kieny et al., 1984). Other developments in the production of wildlife vaccines include the construction of an *Escherichia coli* plasmid that carries the glycoprotein gene (Yelverton et al., 1983), and possible synthetic vaccines (Koprowski, 1988). Anti-idiotypic antibodies have also been shown to induce neutralizing antibodies to rabies virus glycoprotein (Reagan et al., 1983).

Control of insectivorous bat rabies may never be possible, however limiting roosting sites in populated areas and education of the public about the hazards of handling sick bats may be the best method of control (Linhart, 1975). Control of vampire bat rabies and subsequently of cattle rabies has been of limited success. Use of effective bovine vaccines and selective reduction of bat populations with the use of anticoagulant substances or fumigation of bat roosts has been employed (Linhart, 1975).

In any control situation, ongoing reporting, surveillance and border protection are essential (Beran and Crowley, 1983; Steele, 1988). Ecological and social factors are of primary importance when considering a control program (Beran and Crowley, 1983; Fernandes and Arambulo, 1985). Public acceptance of any control program is critical and extensive education programs for health professionals and the public are needed (Beran, 1981).

SECTION I. SEROLOGIC SURVEY FOR RABIES SERUM NEUTRALIZING ANTIBODIES IN RACCOONS (*PROCYON LOTOR*) IN TWO COUNTIES IN IOWA

SUMMARY

Between 1984 and 1988, a study was conducted to evaluate the nature and prevalence of rabies virus neutralizing antibodies in raccoons (*Procyon lotor*) in two counties in Iowa. A total of 985 raccoons were trapped and tagged in Guthrie and Cerro Gordo counties during the spring, summer and fall of each year. Blood samples from 1048 raccoons were collected. Sex, age and other parameters were recorded for each animal. Serum samples were tested for the presence of serum neutralizing antibodies (SNA) by the rapid fluorescent focus inhibition test (RFFIT), mouse serum neutralization test (MSN), and an indirect fluorescent antibody (IFA) technique for detecting immunoglobulin G. The study was designed to investigate the prevalence and nature of SNA in normal raccoons in these Iowa counties. Fifty-one raccoons (5.2%) were found to have SNA by the RFFIT. Thirty-six serum samples were also tested by the MSN, with results correlating well with the RFFIT results. Of 35 raccoons with measurable SNA, six individuals were found to be positive by IFA. In contrast, 2 of 737 (0.27%) of raccoons submitted for rabies testing to the Iowa State University Veterinary Diagnostic Laboratory during the study period were found to be positive for rabies antigen. The disparity between the percentages of animals with SNA and those animals found to be positive by antigen detection tests, suggest that exposure to rabies virus in the wild is greater than indicated by the results from laboratory confirmed cases.

INTRODUCTION

Two epizootics of raccoon rabies in the southeastern and mid-Atlantic areas of the United States have made the raccoon the second most commonly reported rabid wildlife species since 1982 (Baer et al., 1990). Reported cases of raccoon rabies in the United States increased from 62 cases in 1962, to 1820 cases in 1983 (Centers for Disease Control, 1984; National Communicable Disease Center, 1964). Although the number of cases dropped to 1463 in 1988 (Centers for Disease Control, 1989), areas considered to be enzootic for raccoon rabies have continued to enlarge outwardly from initial foci of infection (Jenkins et al., 1988). It is believed that the mid-Atlantic epizootic originated from transplanted raccoons from the southeastern epizootic (Smith et al., 1984). Presently, the majority of rabies cases in raccoons in the United States are limited to these two enzootic areas. In 1988, raccoons from these areas accounted for 99.4% of all cases of raccoons rabies in the United States (Centers for Disease Control, 1989). Outside of raccoon rabies enzootic areas, raccoons are believed to be alternate hosts and not involved in the transmission and maintenance of the disease in wildlife (Smith et al., 1986).

Serologic investigations of the prevalence of serum neutralizing antibodies (SNA) in raccoons have shown that the prevalence is much greater than the point prevalence of the disease based on data from diagnostic laboratories (McLean, 1975). Studies on sera collected outside of epizootic areas in Alabama, Texas, Tennessee, Illinois, and South Carolina have shown seropositive rates ranging

from 0-5.6% (McLean, 1975). Seropositive prevalence rates of 3-12% have been reported in counties bordering an epizootic in Florida 130 miles from the front (McLean, 1975). In contrast, other serologic studies in non-enzootic areas have demonstrated no evidence of rabies; no detectable SNA was reported in Virginia in 1982 on the border of an epizootic area and 100 miles distant (Carey and McLean, 1978), nor in the Great Smoky Mountains in 1979 and 1980 (Rabinowitz and Potgeiter, 1984).

Within areas with enzootic raccoon rabies, or following epizootics, seropositive prevalence rates in raccoons range from 7.2% (McLean, 1975) to 28% (Bigler, 1973). Point prevalence as high as 35% has been reported (McLean, 1975). Serologic studies during the mid-Atlantic epizootic have shown prevalence rates of 14.26% to 18.56% (Centers for Disease Control, 1983 and 1985).

With this evidence, many researchers have concluded that many raccoons develop subclinical forms of rabies (Bigler et al., 1983; Doege and Northrup, 1974; McLean, 1975). There is limited evidence of rare inapparent infection from field and laboratory studies in America, Africa, Asia and Europe (Doege and Northrup, 1974). There is serologic evidence of infection without clinical disease in laboratory experiments in normal foxes, skunks and dogs (Bell, 1975; Parker and Wilsnack, 1966; Sikes, 1962).

The presence of SNA in such studies is difficult to interpret because the type of antibody test is not always reported, actual titer may not be reported, and differing levels of classification of positive animals cloud the interpretation. In

some studies, animals with serum neutralizing (SN) antibody titer <8.0 (Rosatte et al., 1990), or <25.0 (Jenkins et al., 1988) were considered negative.

In addition to the lack of agreement on the threshold value, there are reports of low level false positive SN antibody titers in raccoon sera when using fluorescent antibody based cell culture virus inhibition tests (Barton and Campbell, 1988). Cell cytotoxic factors in the serum have been suggested as the cause (Barton and Campbell, 1988).

The true reason for the prevalence of low level SN antibody titer has yet to be determined. Possibilities include previous exposure to nonfatal infection or a latent state of disease as evidenced by terms such as "abortive disease", "inapparent infection", "subclinical carriers", "asymptomatic carriers", "normal survivor" and "survivor with sequelae" which are found throughout the literature (Doege and Northrup, 1974). Other possibilities for the presence of low level rabies-specific SNA in addition to previous exposure to a sub-lethal virus include non-specific virus neutralizing reactions, current rabies infection, or previous vaccination and release.

Rabies has only been sporadically reported in raccoons in Iowa. Only 70 confirmed cases have been reported since 1950 (Iowa Department of Public Health, 1951-1989). In contrast, evidence from a serologic survey in Iowa in 1971 and 1972 indicates that 7.6% of the raccoons had SNA (Niemeyer, 1973). This information, along with serologic reports of SNA in other areas of the country without enzootic raccoon rabies, raises questions about the disease in raccoons in areas without enzootic raccoon rabies.

The purpose of this study was to investigate the prevalence and nature of SNA in normal raccoons in two counties in Iowa and factors associated with seropositive rates, and compare these data with confirmed positive cases.

MATERIALS AND METHODS

Epidemiologic Analysis of Confirmed Rabies Cases

Data of retrospective epidemiological analysis investigating the number of rabies cases in raccoons were collected from Iowa Annual Rabies Summaries (Iowa Department of Public Health, 1950-1989) and from rabies submission summaries from the Iowa State University Veterinary Diagnostic Laboratory.

Animals

Guthrie County raccoons were trapped over the five year period of 1984-1988 by the Department of Animal Ecology, Iowa State University, Ames, Iowa. Trapping occurred in two ten week periods starting in March and August. Blood samples were collected and the animal's sex, weight and field age were recorded. Ages were determined by tooth extraction, sectioning and cementum annuli analysis. Cerro Gordo County raccoons were trapped over a five month period (March-July) during each of the years 1984-1988 by the Iowa Department of Natural Resources. Samples and data were collected similar to that for Guthrie County. Serum samples were also collected from farm raised raccoons[1]. All sera were heat inactivated (56° C for 30 minutes) and kaolin treated with a 25% kaolin preparation for 30 minutes to absorb non-specific inhibitors. Serum samples were stored frozen (-20° C) until tested.

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[1]Ruby's Fur Farm, New Sharon, IA 50207.

Serum Neutralizing Antibody Titer Determination

Sera were analyzed by the rapid fluorescent focus inhibition test (RFFIT) (Smith et al., 1973) and the mouse serum neutralization (MSN) test (Atanasiu, 1973) for the presence of antibodies against rabies virus. Serial two-fold dilutions were made and titer expressed as reciprocal of the highest dilution capable of reducing the number of rabies infected baby hamster kidney (BHK) cells by 50%, or producing 50% mortality in mice as determined by the Reed-Muench method (Reed and Muench, 1938). Test controls for the RFFIT included uninfected cell controls, back titration of rabies virus challenge dose, negative antirabies sera and titration of positive raccoon antirabies sera. Sera were tested in BHK-21(C-13) cells[2] using the Challenge Virus Standard (CVS-11) virus[3]. Test controls for the MSN test included positive and negative raccoon antirabies sera and 25-50 mouse intracerebral lethal $dose_{50}$ ($MICLD_{50}$) of a CVS rabies virus[4]. Five 13-15 gram female mice[5] per serum dilution were used. Ten mice per dilution were used for the challenge titration.

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[2]Cells originated from the Centers for Disease Control and were supplied to our laboratory by the National Veterinary Services Laboratories, Ames, IA 50013.

[3]Rabies virus from the Centers for Disease Control, Atlanta, GA 30333.

[4]Rabies virus provided by the National Veterinary Services Laboratories, Ames, IA 50013.

[5]Sprague Dawley CF-1 mice, Harlan Sprague Dawley, Inc., Indianapolis, IN 46229.

Indirect Fluorescent Antibody Determination

An indirect fluorescent antibody (IFA) test was performed using an adaptation of a previously described test (Johnson and Emmons, 1980). One-tenth milliliter of a dilution of Street Alabama Dufferin (SAD) virus[4] was added to 0.4 milliliters of a cell suspension of Madin Darby canine kidney (MDCK) cells[6] in eight chamber Lab-Tek cell culture slides[7]. Slides were incubated (37° C) in a humid chamber with 3-5% CO_2. After 72 hours, the supernatant was removed and the cells fixed with acetone (4° C) for 10 minutes, air dried and stored frozen until use. The cell sheet consisted of approximately 30% rabies infected cells. Following a phosphate buffered saline (PBS) rinse, 50 microliters of each dilution of raccoon sera were added to each chamber and incubated (37° C) for 30 minutes in a humid chamber. Slides were washed for 10 minutes in PBS. Each well was filled with 50 microliters of fluorescein isothiocyanate conjugated goat origin antiraccoon immunoglobulin G[8] and incubated (37° C) for 30 minutes. Positive cell control wells were incubated with fluorescein isothiocyanate conjugated equine or bovine origin antirabies globulin[9]. Following a 10 minute wash in PBS, the slides were covered with a 50/50 (V/V) glycerine-

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[6]Cells originated from the ATCC and were supplied to our laboratory by the National Veterinary Services Laboratories, Ames, IA 50013.

[7]Miles Scientific, Division of Miles Laboratories, Inc., Naperville, IL 60566.

[8]Kirkegaard and Perry Laboratories, Inc., 2 Cessna Court, Gaithersburg, MD 20879.

[9]BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, MD 21030.

saline solution, overlaid with coverslips and examined with a fluorescent microscope. Titer is expressed as the reciprocal of the highest serum dilution still exhibiting specific fluorescence. Test controls included uninfected and infected cell controls, negative antirabies sera from farm raised raccoons (RFFIT antibody titer <4.0), and positive antirabies sera (RFFIT antibody titer of 8.0 and 160.0) from raccoons vaccinated with a killed virus vaccine[10]. Optimal dilutions of the reagents were previously determined by checkerboard titration. Presence of non-specific fluorescence prevented serum dilutions of less than 1:10 from being tested.

Analysis of Data

Data were categorized into tables and graphs to study the effects of the factors such as season, sex and age on presence of SNA in the population. Statistical analysis was performed by comparison of proportions of two independent samples or the Chi-Square test (Snedecor and Cochran, 1989).

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[10]Rabguard-TC, Norden Laboratories, Lincoln, NE 68501.

RESULTS

Retrospective Analysis of Confirmed Rabies Cases

Only 70 confirmed cases of rabies have been reported in raccoons in Iowa since 1950 (Iowa Department of Public Health, 1951-1989). Locations of confirmed cases in the state are shown in Figure 1. In Iowa, there were 939 cases of animal rabies confirmed during the study period from a total of 9388 suitable animals of all species submitted for testing. In 1987 the number of cases was significantly higher ($P<.01$) than previous years and 1987 was the peak year for confirmed rabies cases. The striped skunk (*Mephitis mephitis*) is the principle reservoir and accounted for 57.7% of the total number of cases and 97.5% of the reported cases in terrestrial wildlife between 1984 and 1988. Domestic animals accounted for 35.7% of the cases, bats accounted for 5.1%, and other wildlife species accounted for 1.5%. Reported cases by year are shown in Table 1. The relationship between reported skunk cases and the reported cases in domestic animals is well described and is shown in Figure 2.

In Guthrie County, 115 animals of all species were tested during the study period and 8 (7.0%) were found positive. In Cerro Gordo County, 150 animals were tested with 15 (10.0%) positive. The peak year for laboratory confirmed rabies in both counties was also 1987 (Table 2). Year by year positive prevalence rates for these two counties echoed the statewide prevalence rate.

Statewide, the majority of the positive cases were due to skunks which had an overall test positive rate of 79.0%. Year by year testing and positive rates are

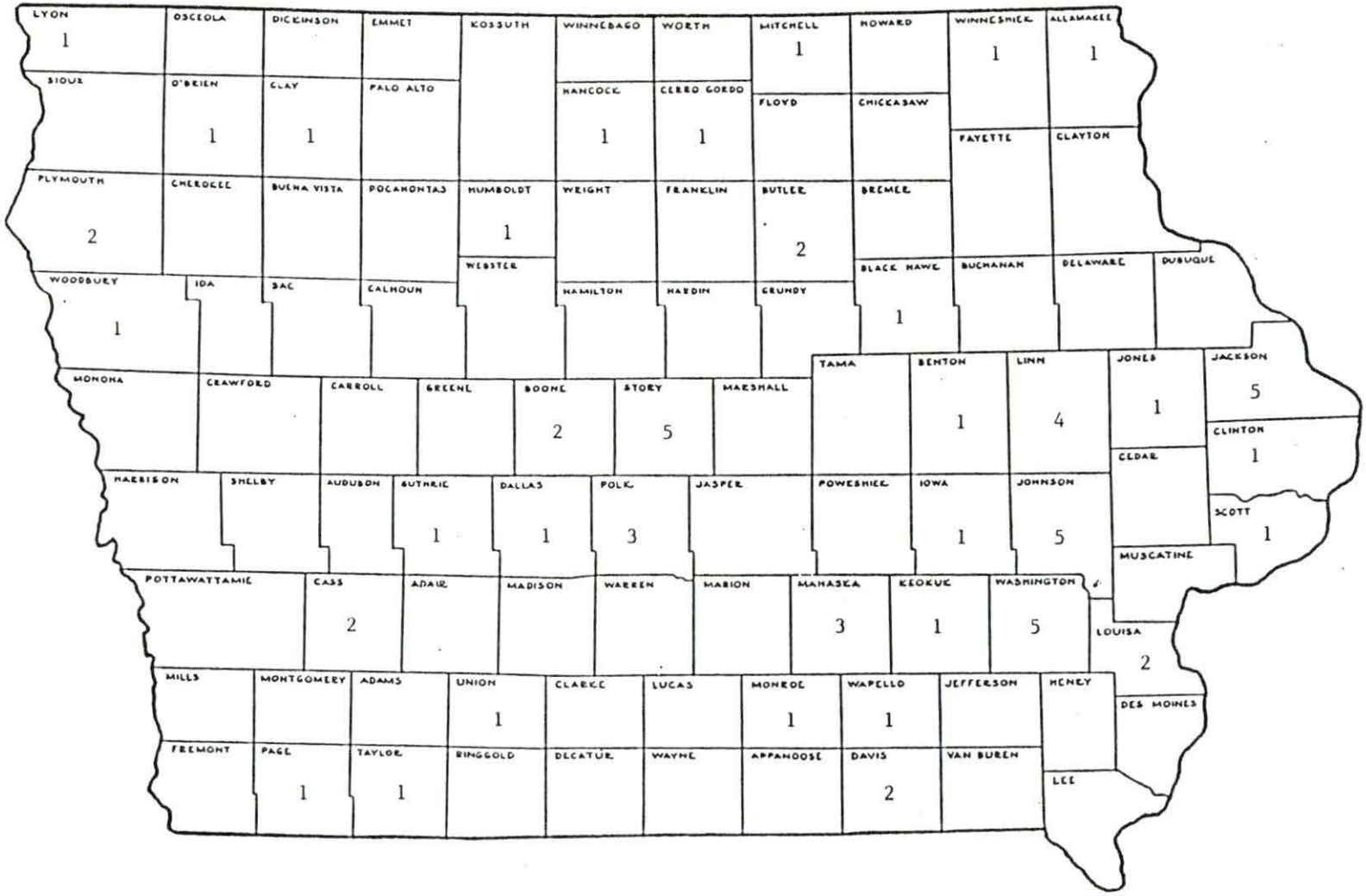

Figure 1. Number of laboratory confirmed cases of rabies in raccoons in Iowa counties for the years 1951-1989

Table 1. Reported cases of rabies in skunks, domestic animals, bats, and wildlife in Iowa for the years 1984-1988

Year	Skunks	Domestic Animals	Bats	Other Wildlife	Total
1984	91	50	8	1	150
1985	95	37	17	1	150
1986	99	84	6	2	192
1987	161**	90	12	8	272**
1988	96**	74	5	0	175**
Total	542	335	48	12	939
Percent	57.7	35.7	5.1	1.5	

** Significantly different ($P<.01$) from the previous year.

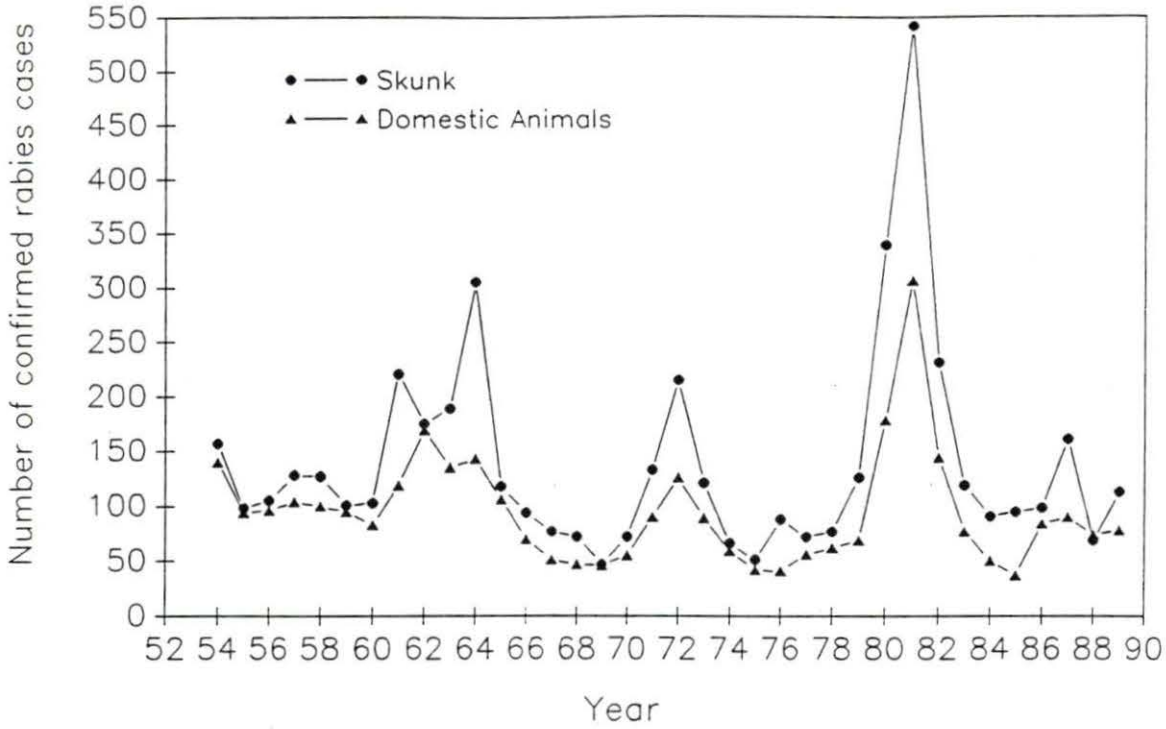

Figure 2. Number of laboratory confirmed cases of rabies in skunks and domestic animals in Iowa for the years 1954-1989

Table 2. Reported number of animals tested for rabies in Cerro Gordo and Guthrie Counties for the years 1984-1988

	Cerro Gordo County			Guthrie County		
Year	Examined	Positive	Percent Positive	Examined	Positive	Percent Positive
1984	31	2	6.5	22	2	2.1
1985	29	2	6.9	19	0	0.0
1986	26	3	11.6	23	1	4.3
1987	37	7	18.9	28	4	14.3
1988	27	1	3.7	23	1	4.3
Total	150	15	10.0	115	8	7.0

Table 3. Reported number of skunks, raccoons and all animals tested for rabies in Iowa for the years 1984-1988

	Skunk			Raccoon		Total All Species		
Year	Examined	Positive	Percent Positive	Examined	Positive	Examined	Positive	Percent Positive
1984	143	91	63.6	146	0	1849	150	8.1
1985	109	95	87.2	163	0	1886	150	8.0
1986	144	99	68.8	188	1	1944	192	9.9
1987	189	161	85.2	113	1	1978	272	13.9
1988	101	96	95.0	127	0	1731	175	10.1
Total	686	542	79.0	739	2	9388	939	10.0

shown in Table 3.

Two cases of rabies in raccoons were reported in the state between 1984 and 1988, from a total of 737 raccoons (0.27%) submitted for testing. In each year except 1987, more raccoons were submitted for testing than skunks. No confirmed cases of rabies in raccoons were found in either of the two counties involved in the study during the sampling period.

Characteristics of Raccoon Samples

Guthrie County

A total of 951 blood samples were collected from 891 wild trapped raccoons during two ten week trapping periods in the spring/early summer and late summer/fall of 1984 through 1988. Fifty-two animals were sampled more than once, eight of which were re-trapped in the same season of the same year. The number of samples collected each year were statistically balanced ($P<.05$). Table 4 shows that with the exception of 1984, the majority of the samples were collected during the second ten week trapping period. Distribution of samples by age group were similar; 53% were from adults and 47% from juveniles less than one year old. The majority of the juveniles (96.9%) were collected during the second trapping. The sex distribution of animals trapped over the 5 year period was 54.3% male and 45.7% female. A larger percentage of the males (37.8%) were trapped during the spring than females (30%). The distribution of age groups within sexes was similar. Table 5 shows the number of samples collected in each sex and age category by year.

Table 4. Number of raccoons trapped in Guthrie County during the years 1984-1988

Year	First Trapping March - July	Second Trapping August - October	Total	Percent By Year
1984	90	91	181	19.0
1985	76	136	212	22.3
1986	70	122	192	20.2
1987	53	154	207	21.8
1988	37	122	159	16.7
Total	326	625	951	100.0

Table 5. Number of raccoon serum samples collected in Guthrie County for the years 1984-1988

	Male		Female	
Year[a]	Adult	Juvenile	Adult	Juvenile
1984	80	27	46	28
1985	50	62	53	57
1986	59	45	54	34
1987	57	51	46	53
1988	42	43	27	47
Total By Age Group	288	228	216	219
Total By Sex	516		435	

[a]Includes 8 animals trapped more than once in a season and 12 animals trapped more than once in a year.

Cerro Gordo County

A total of 97 blood samples were collected from 94 wild trapped raccoons during a five month collection period (March-July) for the years 1984-1988. Three raccoons were sampled twice. Sample size on a yearly basis ranged from a low of 12 in 1984 to 28 in 1985. The largest number of collections were in May and June with 32.0% and 24.7% of the samples collected during these months. All of the raccoons sampled were adults and the majority (69.1%) were male (Table 6).

Serum neutralizing antibody analysis

Sera from 1048 wild trapped raccoons were tested by the RFFIT test for SNA. Fifty-one of 985 raccoons (5.2%) had SN antibody titer >3.0. Titer ranged from 3.2 to 24.2. Sera from 30 farm raised raccoons were tested and none had measurable SN antibody titer.

In order to confirm the results of the RFFIT test, 36 samples (24 with RFFIT antibody titer >3.0, and 12 <3.0) were subjected to the MSN test and correlation analysis. Twenty-three samples were positive by the MSN test with titer ranging from 3.2 to 17.9 (Table 7). The RFFIT test identified one more positive than the MSN test. Results from the two tests were well correlated ($r=0.86$, $P<.01$). Regression analysis shows that overall the RFFIT titer was 1.28 times that of the MSN (Figure 3).

Table 6. Number of raccoons trapped in Cerro Gordo County for the years 1984-1988

Year	Male	Female	Total	Percent By Year
1984	10	2	12	12.4
1985	21[a]	7	28	28.9
1986	12	5	17	17.5
1987	10	7	17	17.5
1988	14	9	23	23.7
Total	67	30	97	
Percent	69.1	30.9		

[a]One animal trapped twice in 1985.

Table 7. Correlation of rapid fluorescent focus inhibition test (RFFIT) antibody titer and mouse serum neutralization (MSN) test antibody titer determinations

MSN Antibody Titer	RFFIT Antibody Titer		
	<3.0	>3.0	Total
<3 .0	12	1[a]	13
>3.0	0	23	23
Total	12	24	36

[a]RFFIT antibody titer of 4.8.

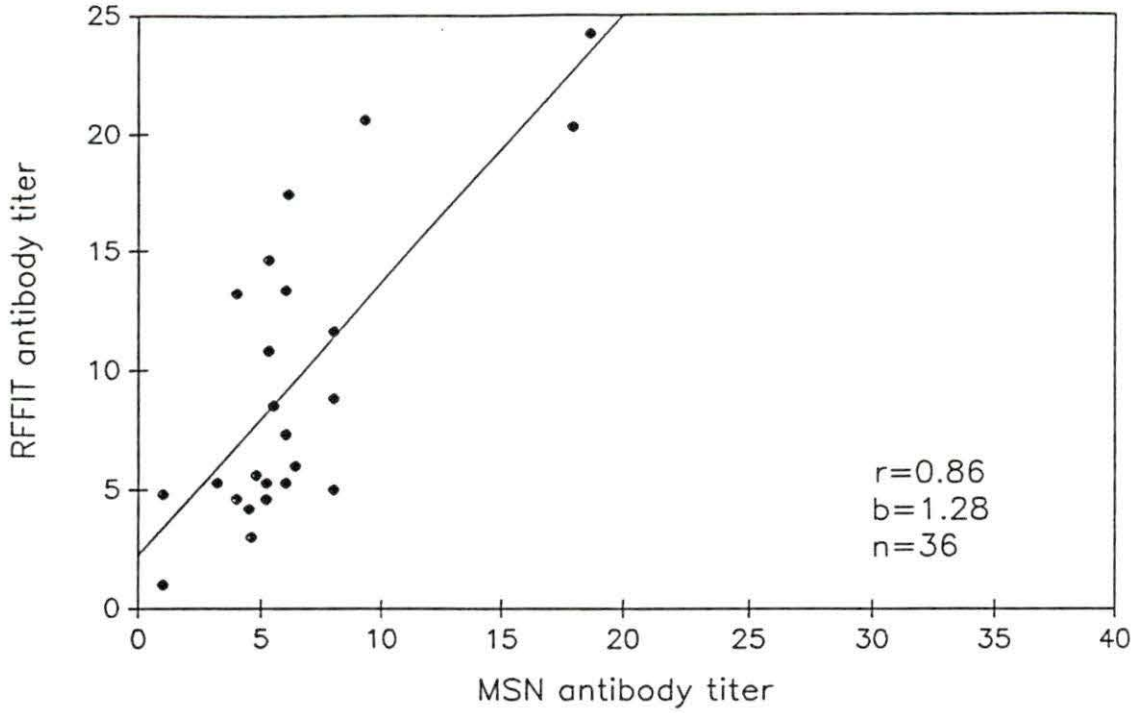

Figure 3. Correlation plot between rapid fluorescent focus inhibition test (RFFIT) and mouse serum neutralization (MSN) test antibody determinations for the sera shown in Table 7

Guthrie County

Forty-eight of 891 animals had SN antibody titer >3.0 by the RFFIT, representing an overall prevalence rate of 5.4%. Of the 52 animals which were sampled more than once, 47 animals with no measurable SNA were re-trapped multiple times, 1 had SN antibody titer >3.0 on trapping dates 6/10/87 and 9/27/87, and 4 animals had SNA in the final sample collected.

Prevalence rates varied among the years, seasons, sexes and age groups. Prevalence rates by year were 4.4% in 1984, 2.1% in 1985, 1.6% in 1986, 9.7% in 1987 and 6.9% in 1988. The rate in 1987 was significantly higher ($P<.01$) than the previous years. Correlation between these prevalence rates and the number of skunk cases in the state is shown in Figure 4.

On a seasonal basis, significantly more ($P<.01$) of the samples from the first trapping period had measurable SNA (8.3%) than from the second trapping period (3.6%) (Table 8). The rate of measurable SNA was significantly higher ($P<.01$), among adults (7.8%) as compared to juveniles (2.3%). All positive juveniles were found during the fall. The prevalence rates were similar among males (5.4%) and females (4.9%). When comparing prevalence rates among season and sex groups for adults, adult males trapped during the spring/early summer trapping showed the highest prevalence rate (9.8%), with adult males sampled during the late summer/fall showing the lowest rate (3.8%) (Table 8). Seasonal rates among adults were not significantly different.

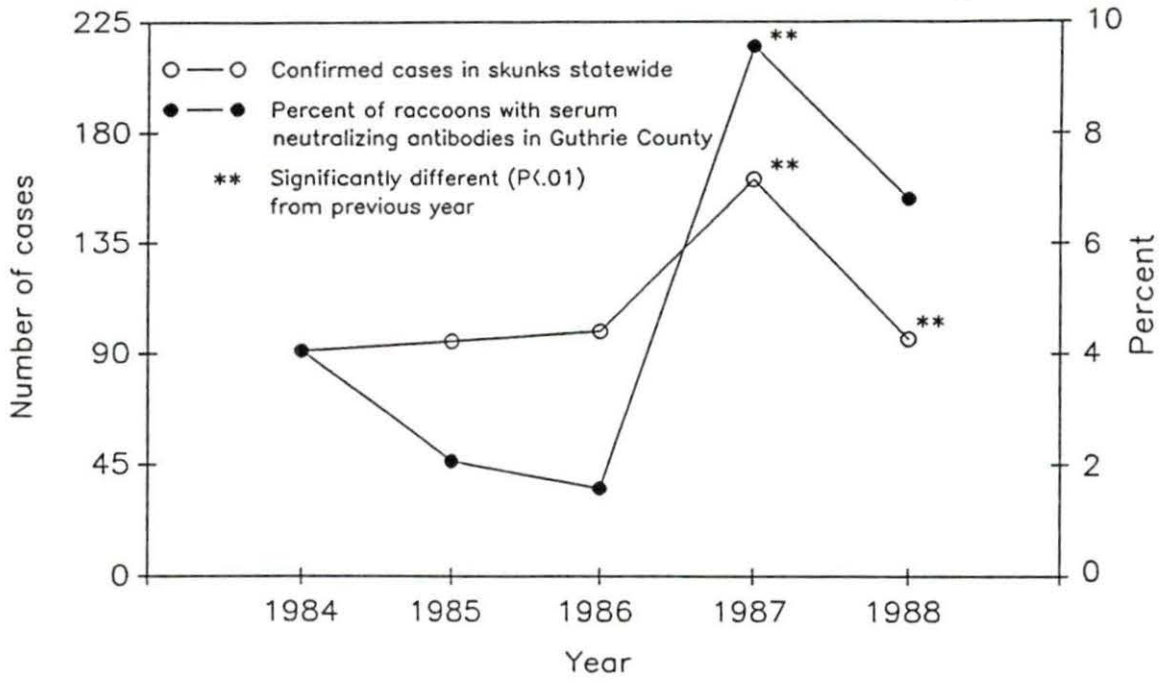

Figure 4. Correlation between laboratory confirmed cases of rabies in skunks in Iowa and serum neutralizing antibody titer as determined by the rapid fluorescent focus inhibition test (RFFIT) in raccoons in Guthrie County for the years 1984-1988

Table 8. Percentage of Guthrie County raccoons with serum neutralization antibody titer >3.0 as determined by the rapid fluorescent focus inhibition test (RFFIT) for the years 1984-1988

Trapping Period[a]	Adult		Juvenile		Total
	Male	Female	Male	Female	
March-July	9.8 (18/183)[b]	7.1 (9/127)	0.0 (0/11)	0.0 (0/3)	8.3 (27/324)**
August-October	3.8 (4/103)	9.3 (8/86)	2.8 (6/217)	1.9 (4/213)	3.6 (22/619)
Total By Sex	7.7 (22/286)	8.0 (17/213)	2.6 (6/228)	1.9 (4/216)	
Total By Age Group	7.8 (39/499)**		2.3 (10/444)		

[a]Eight animals trapped more that once in the same season are not included in these data.

[b]Number of animals in each category shown in parenthesis.

**Significantly different ($P<.01$) from corresponding trapping or age group category.

Cerro Gordo County

Three of 94 raccoon samples had SN antibody titer >3.0 by the RFFIT, representing an overall prevalence rate of 3.2%. Three animals with no measurable SNA were sampled more than once. The three samples with SNA came from two adult males trapped in June, 1988 and from one adult female trapped in April, 1986.

Indirect fluorescent antibody analysis

Sera from 81 wild trapped raccoons from both counties were tested by the IFA test (35 with RFFIT antibody titer >3.0, and 46 <3.0). Insufficient amounts of sera prevented all of those with RFFIT antibody titer from being tested by the IFA. None of the sera with RFFIT antibody titer <3.0 were positive by the IFA test. Six animals were positive for antirabies immunoglobulin G by the IFA test at the 1:10 dilution and three of these at the 1:25 dilution. All of the positive samples were from adult raccoons in Guthrie County. Sex, seasonal distribution, and RFFIT and MSN antibody titers of those animals positive by the IFA test are shown in Table 9.

In an attempt to determine the sensitivity of the IFA test, 11 sequential serum samples (RFFIT antibody titer >3.0) from vaccinated raccoons and raccoons experimentally infected with skunk virus, and 20 sera from farm raised raccoons (RFFIT antibody titer <3.0) were subject to the IFA test and correlation analysis.

Samples with RFFIT antibody titer <3.0 were negative by the IFA test at

Table 9. Guthrie County raccoons with rabies-specific immunoglobulin G as detected by an indirect fluorescent antibody (IFA) technique

Trapping Date	Sex	Age in Years	Weight in kg.	RFFIT[a] Titer	MSN[b] Titer	IFA[c]
08/18/84	M	1	6.8	4.3	ND[d]	Positive
05/30/87	M	1	5.9	10.8	5.3	Positive
06/07/87	M	3	7.2	5.3	5.2	Positive
08/31/87	F	1	5.4	13.3	6.0	Positive
09/10/87	F	4	6.8	6.4	ND	Positive
05/07/88	M	2	6.1	17.6	ND	Positive

[a]Antibody titer as determined by the rapid fluorescent focus inhibition test.

[b]Antibody titer as determined by the mouse serum neutralization test.

[c]Indirect fluorescent antibody results at serum dilution of 1:10.

[d]ND = Not done.

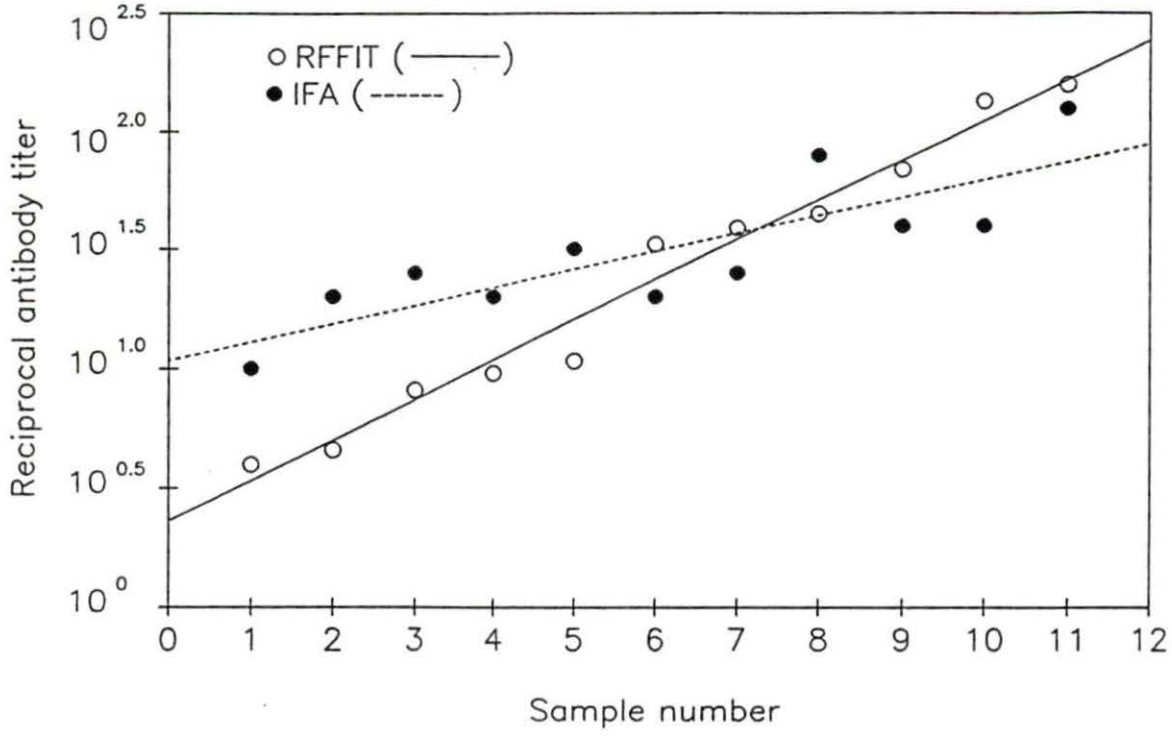

Figure 5. Correlation between rapid fluorescent focus inhibition test (RFFIT) and indirect fluorescent antibody (IFA) antibody titers in sera of raccoons with known exposure to rabies antigen. Samples are paired by increasing order of RFFIT antibody titers

the 1:10 dilution. Samples with measurable RFFIT antibody titer were positive by the IFA test. Results of the two tests were correlated ($r=0.76$, $P<.01$).

Regression analysis comparing the IFA titer to the RFFIT titer demonstrates that the relative IFA titer changes as the RFFIT titer increases. At low level RFFIT titer (<40.0, $10^{1.6}$), the IFA titer is higher and at higher RFFIT antibody titer the IFA titer is lower as shown in Figure 5.

DISCUSSION

Even though clinical rabies in raccoons is uncommon in the central United States, it is important to understand the role of the raccoon in areas where skunk rabies is enzootic in order to fully evaluate the public health importance of exposure to raccoons. Before the 1950s, rabies in raccoons was only sporadically reported in the United States. Epizootics in the southeast and mid-Atlantic regions over the last 40 years are indicative of an expansion of the raccoon adapted variant virus and the disease is continuing to expand outwardly from the enzootic centers (Centers for Disease Control, 1985; Jenkins et al., 1988). Should raccoon rabies ever become enzootic in areas with rabies in other species, the entire transmission/exposure cycle would become complicated. It is possible that raccoon rabies could expand across the whole country, or new epizootics could develop. Therefore, a basic understanding of the existing prevalence in non-enzootic areas is critical.

Since the early 1960s, Iowa has consistently been among the states reporting the highest total number of rabies cases yearly in the continental United States (Centers for Disease Control, 1977, 1987, 1988 and 1989). In Iowa, the sylvatic cycle of transmission of rabies is maintained in wild animals (Beran, 1981; Pacer et al., 1985). Although rabies is diagnosed in many species of animals in Iowa each year, striped skunks have dominated the total number of reported cases since 1953 and are considered the primary reservoir (Beran, 1981; Iowa Department of Public Health, 1953-1989). Recent studies using monoclonal

antibodies have shown that only the skunk and bat variants have been identified in Iowa (Smith et al., 1986). Dog variants were probably present until widespread use of domestic animal vaccines after World War II significantly reduced the number of dog cases in the United States (Pacer et al., 1985).

This retrospective study on the specimens submitted to the Veterinary Diagnostic Laboratory for rabies examination helps confirm the role of the skunk as primary reservoir host in this state. When compared to all other species, the rate of rabies positive skunks per number of samples submitted (79.0%), is significantly higher ($P<.01$) than for any other species. Cases in other species are the result of spill-over from the primary host. Numbers of cases in other animals parallels numbers of cases in skunks. Total number of cases in the state were not significantly different from 1984 to 1986, but did increase significantly ($P<.05$) in 1987. A similar trend was seen in the two study counties, demonstrating that on a smaller scale, these two counties are representative of the occurrence of rabies in the state. The increase in 1987 was expected because of the 7-9 year cyclical nature of rabies in the state. The small percentage (0.27%) of confirmed cases of rabies in raccoons in the state during the study period provides evidence that rabies is not maintained in raccoons.

The distribution of animals trapped during this study is consistent with previous studies involving the trapping of raccoons in the wild (Bigler et al., 1973; Hubbard, 1985), indicating this sample correlates with other random trapping surveys. Sampling of more males than females was observed in both of the study counties. Increased trapping of juveniles occurred during the fall trapping and is

expected as young raccoons begin to leave the family unit and enter the general population.

Prevalence rates for animals with SNA in this population varied among age groups, season and year. There were no significant differences between the sexes, adult age categories, or between weight group categories. This is to be expected because adult animals in the population should have equal chances of exposure to the virus.

Distribution of positive animals varied among the adults and juveniles; there were significantly more adults than juveniles with SNA. This is consistent with length of time in the population being correlated with risk of exposure.

The limited time frame for sample collection in this study does not allow detailed seasonal analysis. The significant decrease in prevalence rates from the spring to the fall trapping coincided with the large number of negative juveniles entering the population during the second trapping period. In raccoon rabies enzootic areas it is believed that rises in prevalence of SNA in the spring and fall are associated with animal contact during breeding activities and movements prior to the winter denning period.

There was a significant rise in the prevalence rate in raccoons in 1987. This increase is correlated with the significant increase in the number of confirmed laboratory cases in the skunk in the state and the total number of cases occurring in each county during that year. Although the number of skunk cases dropped significantly the following year, the prevalence rate for positive raccoons by the SN test was not significantly different. Seropositive raccoons in 1988 were equally

balanced between the sexes and were higher in adults. Persistence of SNA in animals exposed the previous year may account for the rate remaining high. The one animal that was re-trapped and remained positive 109 days after the first positive sample provides evidence for the extended survival of animals with SNA in the wild. Previous studies have shown that re-trapped wild raccoons in enzootic areas can maintain SNA for 37 months (Bigler et al., 1983). Since these were wild animals, exposure or re-exposure to the virus is uncertain.

Conclusions about the prevalence rate of SNA in this study come mainly from the Guthrie County data because of the larger sample size. Prevalence rates for Guthrie County (5.4%) and Cerro Gordo County (3.2%), were not significantly different ($P<.01$). Therefore, results from Guthrie County could be extrapolated to Cerro Gordo County.

We must be cautious however in interpreting too much from a population with such low levels of SNA. There are other possibilities for virus neutralizing ability of sera from wild trapped raccoons. It is possible that antibodies directed against an agent other than rabies virus were the cause of the inhibition. Perhaps another rhabdovirus shares nucleoprotein antigens which cross react with the rabies virus in the test systems. The possibility exists of positive animals having been previously vaccinated and released into the wild. It is also possible that some factor in the sera was causing non-specific neutralization and the samples were falsely positive. There is little published information concerning non-specific SNA in wildlife, yet many studies exclude animals with low level SNA as falsely positive.

In this study, three different tests were used to assess rabies antibody activity in the wild trapped raccoons. The RFFIT and MSN tests are based on the measurement of the ability of factors in the serum to inhibit the growth of challenge virus in cell cultures as detected by fluorescent antibody staining or the pathogenesis of the virus in mice. The mouse test has been the accepted standard for comparing other antibody detection tests. It is well reported that the neutralizing ability of serum samples is due to the presence of virus-specific neutralizing antibodies.

There is little evidence of false positive reactions with the MSN test. There are reports of false positive levels of 0.8% in studies with pre-vaccination and post-vaccination sera from people (Larsh, 1965; Thomas et al., 1963). Considering the past performance of the MSN test, the most likely conclusion is that the SN antibody titers observed in this study represent specific virus neutralizing activity.

Statistical analysis of the data from this study indicates that the RFFIT and MSN tests correlate well. The RFFIT antibody titer was on the average 1.28 times higher that the MSN antibody titer and identified one more positive than the MSN test. The RFFIT test has been shown to be less specific than the MSN test (Smith et al., 1973). Correlation between the RFFIT and MSN tests in this study provides evidence that lack of specificity in the RFFIT test was no different than previously reported.

The third test was the IFA technique to detect immunoglobulin G. Using the results from known samples, there was good accord between the IFA and the

RFFIT test in recognizing positive and negative reactors. The results of the positive IFA tests in the wild trapped raccoons help to confirm that exposure to rabies antigen was occurring in the sample area. The number of positive samples by the IFA is too small to support detailed analysis of prevalence rates among different years, seasons, sexes, or age groups, but it is important to note that four of the six positive samples were from raccoons trapped in 1987, the peak year for rabies in each county and the state, and that no animals which were positive by the IFA test were negative by the RFFIT or MSN test.

Although the overall correlation between the two tests in known sera with rabies-specific RFFIT antibody titer is good, at low level SN antibody titer (<40), the IFA titer was usually higher than the RFFIT titer and at higher SN antibody titer the IFA titer was usually lower. This indicates that the IFA is more sensitive in sera with low antibody titer. Similar differences in sensitivity as SN antibody titer changes have been reported (Grandien and Espmark, 1975).

The IFA test measures antibody binding rather than specific virus neutralizing antibody. The RFFIT and IFA tests do not measure identical spectrum of antibodies; the binding in the IFA test is with the nucleocapsid rather than with glycoprotein as in the RFFIT (Campbell and Barton, 1988). Discrepancies between fluorescent antibody based tests and other antigen-antibody binding tests in sera with low level SNA have been reported (Barton and Campbell, 1988). Technical difficulties could also account for differences. Extra washing steps in the IFA test could disassociate antibodies with low affinity.

Non-specific fluorescence at low serum dilutions does not allow titration of

sera by this IFA technique at less than the 1:10 dilution. This prevents direct comparison between the IFA and SN based tests in samples with low level titer. All of the wild trapped animals had SN antibody titer <25, and the majority of the samples had an antibody titer <10 by both the RFFIT and MSN tests. Inconsistent results or poor correlation of the IFA test with the MSN test has been reported (Grandien, 1977, Grandien and Espmark, 1975; Thomas et al., 1963), as has false negative rates 14.3% in sera with low level SN antibody titer (Peck, 1966).

Another possibility is that samples positive by the SN antibody based tests, but negative by the IFA, could be due to another class of antibody. If animals had been recently exposed to rabies, and only immunoglobulin M was present in the sera, the IFA test would be negative. The time period that this would occur would be limited to a few days, making it unlikely that many animals would have been sampled during this period.

The need for further evaluation of the IFA test is demonstrated, yet the IFA positive results help to confirm that animals with SNA have been exposed to rabies antigen.

If positive antibody titer represent post-exposure response, then questions exist as to whether the animals were in the process of developing clinical rabies, or were exposed and experiencing inapparent infection and halted progression of the disease. There is evidence of inapparent infection in raccoons in experimental challenge studies with a skunk rabies isolate (Section II. of this thesis). Raccoons developed rabies-specific SNA without dying when given 63,200

mouse intracranial lethal dose$_{50}$ (MICLD$_{50}$) intramuscularly, suggesting that inapparent infections do occur. Further studies are needed to determine if the animals in the wild which are positive by serologic tests, had subclinical non-fatal rabies infections, had recovered from clinical disease, or were destined to die of clinical rabies.

It appears from this study that there can be small numbers of raccoons with SNA in the wild in areas without enzootic raccoon rabies. Many of these animals have very low titer. Also, the number of animals statewide confirmed as rabies positive in the laboratory do not reflect the number of animals in the wild with SNA. Previous reports of the prevalence of SNA in raccoons has raised questions about rabies in raccoons in non-enzootic raccoon rabies areas. This study suggests that these questions are valid. The results of these tests demonstrate we must be cautious in the interpretation of serologic studies in wildlife and extrapolating from these data to make definitive statements about the prevalence of the disease in wildlife species. The RFFIT, MSN and IFA tests demonstrate that exclusion of all animals in serologic studies with titer less than a certain level is not justified. Such information could be helpful in the interpretation of seropositive prevalence rates in future serologic surveys. In addition, specific conclusions can be drawn from this study: 1) If those animals with SNA truly represent animals previously exposed to rabies virus, then it appears that a percentage of the raccoons in these counties are able to thrive and appear clinically normal after exposure. 2) With such a small percentage of raccoons having SNA, the raccoon does not play a significant role in the epidemiology of

the disease in these counties. 3) A minimum of 17% of the sampled animals with low level SN antibody titer (<25.0) were shown to have rabies-specific immunoglobulin G. 4) If an enzootic of raccoon rabies entered these counties, the immune status of so few raccoons would not affect the occurrence of an epizootic of raccoon rabies.

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ACKNOWLEDGEMENTS

The author thanks Dr. William Clark and associates from the Department of Animal Ecology, Iowa State University, for providing the Guthrie County primary data and Ron Andrews and associates from the Iowa Department of Natural Resources for the Cerro Gordo County data.

SECTION II. EXPERIMENTAL INFECTION OF RACCOONS (*PROCYON LOTOR*) WITH RABIES VIRUS OF SKUNK ORIGIN

SUMMARY

To determine raccoon (*Procyon lotor*) susceptibility and serum neutralizing antibody response to a skunk salivary gland rabies virus, nine raccoons were inoculated with a rabies virus isolated from a naturally infected Iowa striped skunk (*Mephitis mephitis*). The raccoons were divided into three groups of 3 animals and on day 0, varying dilutions of a virus suspension; $10^{2.4}$,$10^{3.4}$,$10^{4.8}$ mouse intracerebral lethal dose$_{50}$ (MICLD$_{50}$), were administered into the masseter muscles of each animal. Three control animals received only diluent. Animals were observed daily and saliva and sera were collected on post-inoculation days 35, 63 and 92 for virus isolation and determination of serum neutralizing antibody titer. All animals survived the 92 day observation period. One animal developed slowly progressive localized neurologic signs in the front legs beginning 13 days after challenge, yet continued to function without other clinical signs throughout the observation period. Two of the three animals receiving the highest inoculum developed serum neutralizing antibodies (SNA). Rabies virus was not detected in the saliva of any raccoon. On day 92, a suspension of New York City (NYC) strain rabies virus in fox salivary glands ($10^{3.2}$ MICLD$_{50}$) was similarly administered to all raccoons. Two of nine of the animals previously inoculated with the skunk virus survived the NYC virus challenge which killed all controls. Animals that survived the challenge were the ones which had developed SNA.

The results of the first inoculation demonstrate that given significant levels of skunk rabies virus, raccoons can survive at least 92 days and 22.2% of the

animals developed SNA. The development of SNA appears to be dose related. The results of the second inoculation demonstrate that animals with SNA are capable of withstanding subsequent challenge that is fatal for seronegative raccoons.

INTRODUCTION

In regions with enzootic wildlife rabies, clinically normal animals may have serum neutralizing antibodies (SNA) against rabies (Baer, 1975; Constantine, 1966a; Gough and Niemeyer, 1975: McLean, 1975). Serologic surveys of wild trapped raccoons in the United States have found seropositive prevalence rates ranging from 0 to 5.6% in non-enzootic raccoon rabies areas (McLean, 1975) and from 3 to 28% in areas with enzootic raccoon rabies (Bigler et al., 1983; Jenkins et al., 1988). These findings raise the question of survivability of raccoons exposed to different rabies virus variants in the field.

In experimental work designed to study species susceptibility, skunk isolates of rabies virus have been studied in foxes and skunks (Parker and Wilsnack, 1966). Foxes and skunks are equally susceptible to challenge with the skunk virus with as little as 10^2 mouse intracerebral lethal dose$_{50}$ (MICLD$_{50}$) causing 83% mortality in both species.

In experimental studies with virus strains other than the skunk virus, raccoons were found to be 1000 times more resistant than foxes to a challenge with a fox salivary gland isolate (Sikes and Tierkel, 1961). As few as $10^{2.2}$ mouse lethal dose$_{50}$ (MLD$_{50}$) produced 50% mortality, but $10^{4.2}$ MLD$_{50}$ produced only 72% mortality in raccoons. In a separate challenge study in raccoons in Europe, the fatal intramuscular dose was found to be 10^3 MICLD$_{50}$ of red fox virus (Artois et al., 1989). In other studies, raccoons remained clinically normal for 104 days (day of euthanasia) following challenge of $10^{3.4}$ MLD$_{50}$ Mexican free-tailed bat

virus (Constantine, 1966a), and for 243 days (day of euthanasia) following bites by rabid red bats (Constantine and Woodall, 1966). These data indicate that raccoons are somewhat resistant to certain strains of rabies virus. In contrast, a relatively large inoculum of $10^{5.5}$ $MICLD_{50}$ of a Mexican dog virus has been shown to be 100% fatal in raccoons (Rupprecht et al., 1986).

Serologic studies in other species have shown that foxes can survive an experimental challenge with fox virus, develop SNA, and remain clinically normal (Sikes 1962). Still, other skunks and foxes which subsequently died from challenge with fox and skunk rabies virus, also produced SNA prior to death (Sikes, 1962; Charlton et al., 1987). Dogs which have remained clinically normal, or have recovered from clinical illness have also developed SNA following experimental exposure to an Ethiopian rabies virus (Fekadu et al., 1982). In contrast, human beings do not generally develop SNA until 7-10 days after the onset of clinical signs (Hattwick and Greg, 1975).

There are a limited number of reports of the development and protective nature of SNA in raccoons. Clinically normal raccoons have been shown to develop SNA when experimentally bitten by rabid red bats (Constantine and Woodall, 1966). Raccoons have been inoculated with a salivary gland suspension from coyotes previously infected with Mexican free-tailed bat virus and have developed SNA, while remaining clinically normal (Constantine, 1966a and 1966b). In several challenge studies following oral vaccination with a vaccinia-rabies glycoprotein recombinant virus vaccine, not all raccoons with SNA survived challenge (Brown and Rupprecht, 1990; Rupprecht et al., 1986 and 1989).

The above observations have led some to conclude that the development of SNA is not related to survival in experimental challenge studies, nor is the presence of SNA an indication of protection (Artois et al., 1989, Rupprecht et al., 1986).

Raccoon susceptibility to the skunk virus is unknown and little is known about the pathogenicity of the skunk rabies virus in raccoons. This study was designed to investigate the behavior of an Iowa skunk isolate of rabies virus in raccoons in order to determine species susceptibility and serum neutralizing (SN) antibody response.

MATERIALS AND METHODS

Virus Inoculum and Titration

The rabies virus suspension consisted of a homogenate of salivary glands from a naturally infected striped skunk submitted to the Iowa State University Veterinary Diagnostic Laboratory. Mandibular salivary glands were ground with mortar and pestle in 30 milliliters of diluent consisting of 2% normal horse serum in water, pH 7.6, containing 500 units of penicillin and one milligram of streptomycin per milliliter. The suspension was stored at -70° C until use.

The virus suspension was titrated by intracranial inoculation in mice (Koprowski, 1973). Groups of ten 13-15 gram female white mice[1] were inoculated intracerebrally with 0.03 ml of each virus dilution and observed for 21 days. 50% mortality endpoint calculations were made using the Reed-Muench formula (Reed and Muench, 1938).

Animals

Twelve adult farm-reared raccoons[2] were housed individually in stainless steel cages, fed daily and given water ad lib. The animals were housed in an isolation facility, treated for parasites, identified by ear tags and weighed. Each animal was tested for rabies SNA 14 days prior to, and on the day of inoculation. Animals were randomly divided into four treatment groups of 3 animals each.

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[1]Sprague Dawley CF-1 mice, Harlan Sprague Dawley, Inc., Indianapolis, IN 46229.

[2]Ruby's Fur Farm, New Sharon, IA 50207.

Inoculation Procedure and Sample Collection

Animals were anesthetized with a mixture of 2 mg/kg ketamine hydrochloride[3], 2 mg/kg xylazine[4], and 0.2 mg/kg butorphanol tartrate[5] administered intramuscularly. The rabies inoculums consisted of 1 milliliter of the skunk salivary gland suspension diluted to titer of $10^{2.4}$,$10^{3.4}$,$10^{4.8}$ $MICLD_{50}$/ml as determined by simultaneous intracerebral inoculation of mice. Each of three raccoons was inoculated with 0.5 ml of the virus suspension dilution bilaterally in the masseter muscles on day 0. Three animals received only diluent. Raccoons and mice were inoculated within one hour of dilution of the virus. Virus suspensions were held in an ice bath until inoculation.

Animals were similarly anesthetized on days 35, 63 and 92, weighed, and blood samples were collected by jugular vein or cardiac puncture. Saliva samples were collected by swabbing the oral mucosa and tonsilar area with a sterile cotton swab.

On day 92, a suspension of New York City (NYC) strain virus[6] in fox salivary glands ($10^{3.2}$ $MICLD_{50}$) was similarly administered to all raccoons. Animals were observed daily for changes in behavior. Sera were collected at the

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[3]Ketaset, Bristol Laboratories, Syracuse, NY 13220.

[4]Rompun, Haver-Lockhart Laboratories, Shawnee, KS 66203.

[5]Torbugesic, Bristol Laboratories, a division of Bristol-Myers Co., Syracuse, NY 13221.

[6]Rabies virus obtained from the National Veterinary Services Laboratories, Ames, IA 50013.

time of death. Rabies deaths were confirmed by fluorescent antibody[7] staining of acetone fixed impression smears of brain tissue (Dean and Abelseth, 1973).

The NYC virus challenge level for the 12 animals in this study was determined from a titration study in six raccoons. A raccoon lethal $dose_{50}$ of $10^{1.8}$ $MICLD_{50}$ of the NYC virus was determined.

Serum Neutralizing Antibody Titer Determination

Sera were analyzed in triplicate by the rapid fluorescent focus inhibition test (RFFIT), (Smith et al., 1973) for the presence of antibodies against rabies virus. Serial five-fold dilutions were made and the titer expressed as the geometric mean of the reciprocal of the highest dilution capable of reducing the number of rabies infected cells by 50% as determined by the Reed and Muench method (Reed and Muench, 1938). Test controls for the RFFIT included uninfected cell controls, back titration of rabies virus challenge dose, negative antirabies sera and titration of positive raccoon antirabies sera from raccoons previously vaccinated with a killed vaccine[8]. Sera were tested in baby hamster kidney (BHK-21(C-13)) cells[9] using a Challenge Virus Standard (CVS-11) virus[6].

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[7]Fluorescein isothiocyanate conjugated equine or bovine origin antirabies globulin, BBL Microbiology systems, Becton Dickinson and Co., Cockeysville, MD 21030.

[8]Rabguard-TC, Norden Laboratories, Lincoln, NE 68501.

[9]Cells originated from the Centers for Disease Control and were supplied to our laboratory by the National Veterinary Services Laboratories, Ames, IA 50013.

Virus Isolation

Saliva swabs were placed in one milliliter of Glasgow minimum essential medium[10], kept in an ice bath following collection, and stored frozen (-70° C) until tested. Each sample was tested for the presence of rabies virus in murine neuroblastoma cells[11] (Webster and Casey, 1988). Skunk and NYC virus infected cells were used as positive controls.

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[10]Gibco Laboratories, Grand Island, NY 14072.

[11]Cells originated from the Centers for Disease Control and were supplied to our laboratory by the National Veterinary Services Laboratories, Ames, IA 50013.

RESULTS

Skunk Virus Inoculation

None of the animals developed fatal rabies. Raccoon #2 developed a tic in the left front leg on day 13 which slowly progressed into a bilateral twitch of the front legs and shoulders, seen when the animal was in a sitting position. In all other aspects, this animal behaved normally. No adverse reactions were observed in any of the other 11 raccoons. Virus was not detected in any of the saliva samples. All 12 animals continued to gain weight throughout the 92 day observation period. Two of the nine virus inoculated animals had developed SNA by day 35 and remained positive on day 63. Only one animal had SNA by day 92. Table 1 gives the SN antibody levels for each raccoon.

NYC Virus Challenge

Two of the nine animals previously inoculated with the skunk virus survived the challenge. All controls (3/3) died following challenge. Challenged raccoons first showed clinical signs from 11 to 21 days post challenge. Sudden death was seen in three raccoons. Of those raccoons showing clinical signs there were variations, but most animals followed a pattern. Animals had a short period of increased alertness or apprehension (less than 24 hours), followed by extremely aggressive behavior. All animals showing aggressive behaviors died within a few hours of the onset of these signs. Several animals were observed to drink during this phase.

Table 1. Rabies virus-neutralizing antibody titer and response to challenge with two rabies viruses in raccoons

Group	Raccoon Number	Inoculum[a] $MICLD_{50}$	RFFIT[b] Antibody Titer DPC 35	DPC 63	DPC 92	Response to Challenge[c]	RFFIT[d]
A	5		<4.0	<4.0	<4.0	D(15)	<4.0
	11	235	<4.0	<4.0	<4.0	D(12)	<4.0
	12		<4.0	<4.0	<4.0	D(17)	<4.0
B	1		<4.0	<4.0	<4.0	D(21)	31.5
	3	2,400	<4.0	<4.0	<4.0	D(18)	<4.0
	10		<4.0	<4.0	<4.0	D(21)	<4.0
C	2[e]		7.1	4.1	<4.0	S	
	7	63,200	<4.0	<4.0	<4.0	D(15)	32.0
	9		27.6	29.4	24.3	S	
D	4		<4.0	<4.0	<4.0	D(14)	8.6
	6	Controls	<4.0	<4.0	<4.0	D(15)	<4.0
	8		<4.0	<4.0	<4.0	D(15)	5.5

[a]Groups were challenged i.m. with 1 ml of a skunk rabies virus suspension. Controls received 1 ml diluent.

[b]Virus-neutralizing antibody titer as determined by the rapid fluorescent focus inhibition test (RFFIT) at specified number of days post challenge (DPC).

[c]All animals were challenged i.m. on DPC 92 with 1 ml ($10^{3.2}$ $MICLD_{50}$) of NYC rabies virus. S=Survived, D=Died. Day of death following NYC challenge is shown in parentheses.

[d]Virus-neutralizing antibody titer on day of death.

[e]This raccoon developed mild peripheral neurologic signs evident since day 13 following initial inoculation.

None of the 7 animals which had not developed SNA following the skunk virus inoculation survived the NYC virus challenge. Both animals that had previously developed SNA survived. Table 1 gives the post challenge results for each raccoon.

DISCUSSION

Many factors are at work in survivability and transmission of rabies in different species. The geographically restricted, species specific, single reservoir association of rabies virus is well known (Smith et al., 1986). In addition, each animal species seems to have an inherent level of resistance or susceptibility to any rabies virus (World Health Organization, 1973). Individual animal responses to virus challenge complicate the pathogenesis even further.

It is also recognized that each species adapted virus behaves differently in alternate hosts. In wildlife species, the variability of survival following rabies challenge with different virus variants is well documented (Sikes, 1962, Parker and Wilsnack, 1966; Sikes and Tierkel, 1961). Differing threshold levels of minimum virus capable of producing 100% fatality appear to exist. Other studies have verified how the route of exposure and method of challenge preparation can effect the pathogenicity (Soulebot et al., 1982). These factors, in addition to unknown factors in the field, add to the mystery of nonfatal rabies infection.

Previous experimental raccoon inoculation studies have shown that they are relatively resistant to experimental infection with fox virus (Sikes and Tierkel, 1961). Deaths in 100% of the animals was not seen in any dilution up to $10^{4.2}$ MLD_{50}. This study demonstrates that raccoons are resistant to inoculation with considerable amounts of skunk virus. The raccoons in this study were given a higher dose of skunk virus than was previously shown to be lethal in both skunks and foxes (Parker and Wilsnack, 1966).

Only one animal developed a neurologically related clinical sign; a slowly progressing twitch in the forelegs and shoulder area. It is uncertain if rabies virus was involved with the development of this sign. Further examination of this animal's neurologic system at the time of necropsy is planned.

Serologic surveys in both raccoon rabies enzootic areas and areas with other species as reservoir hosts have found that normal raccoons have SNA. This study demonstrates that experimentally infected animals can develop SNA and appear clinically normal. In this case 22.2% (2/9) of the animals receiving the skunk virus developed rabies-specific SNA. These two animals were both from a group of 3 raccoons that had received $10^{4.8}$ $MICLD_{50}$ of the virus. The development of SNA in these two animals provides supportive evidence of immune system infection without disease. Development of SNA in raccoons appears to be related to dose of virus received. Similar results have been reported in skunks (Charlton et al., 1987).

Virus was not detected in any of the saliva samples. This is not unexpected because in other species, shedding of virus for more than a few days prior to the onset of clinical signs has not been reported and the route to the salivary glands is via the central nervous system (Schneider, 1975).

Because of the similarity of the range distributions of striped skunks and raccoons in the United States, occasional exposure of raccoons to rabid skunks is likely. Although there is considerable variation in the amount of virus rabid animals excrete in their saliva, large amounts of virus can be excreted (Sikes, 1962; Parker and Wilsnack, 1966; Vaughn, 1963 and 1965). In skunks, the range

is from zero to 10^4 $MICLD_{50}$/0.03 ml in saliva swab tests in skunks given a fox salivary gland isolate, and up to 10^6 $MICLD_{50}$/0.03 ml in skunks induced to salivate with pilocarpine (Sikes, 1962). Up to $10^{5.3}$ $MICLD_{50}$/0.03 ml is reported when the skunks infected with a skunk salivary gland isolate are induced to salivate with pilocarpine (Parker and Wilsnack, 1966). Such levels of virus excretion allows transmission with very small amounts of saliva.

With all of the animals in this study surviving the inoculation with skunk virus, it appears that inapparent infections in exposed raccoons are possible. It would have been unusual for any of the animals inoculated with the skunk virus to have come down with clinical rabies after more than 92 days. Survival of these nine raccoons provide evidence that exposure of raccoons to rabies virus in the wild may be wider than previously believed.

The results of the NYC virus inoculation indicate that animals previously exposed to the skunk virus can survive lethal challenge. It appears that the primary factor involved are SNA.

In addition to SNA, there are several suggested immune effector mechanisms involved in limiting rabies virus infection (MacFarlan, 1988). These include 1) mechanisms for the destruction of infected cells such as complement mediated cell lysis, cytotoxic T cells, or other cell mediated mechanisms; 2) prevention of cell to cell virus spread by sequestration of infective complexes by phagocytic cells or release of interferon or other soluble mediators. Although a protective role of cell mediated immunity has been previously reported (Wiktor et al., 1985), the absence of protection from the NYC virus challenge in all animals

without measurable SN antibody response, demonstrates that other immune mechanisms appear to be of limited importance in protection. Further studies into the mechanism of protection are required.

Survival of some of the raccoons following the NYC virus challenge is an indication that the challenge was not overwhelming. Studies are planned to follow survivors and monitor the SN antibody response.

The apparent species resistance to inoculation with skunk virus contrast previous reports that raccoons as a species are highly susceptible to rabies virus (World Health Organization, 1973), yet the results of the challenge with the NYC strain supported this hypothesis. It appears that the strain of the virus is a particularly important factor when considering raccoons susceptibility.

Both animals that had developed SNA survived the NYC virus challenge, including the animal whose titer dropped below 4.0. Similar survival results have been observed in foxes which developed SNA following inoculation with fox virus and survived a second challenge which killed all controls (Sikes 1962). In general, correlation between protection from challenge and SN antibody titer are fairly good (MacFarlan, 1988). In contrast, lack of correlation between SNA and protection in raccoons with vaccinia-rabies glycoprotein recombinant virus vaccine induced SNA has been observed (Brown and Rupprecht, 1990; Rupprecht et al., 1989). Varying susceptibilities of raccoons to challenge viruses of different host origin, and efficacy of recombinant vaccines may account for the conflicting data. In any host, absolute correlation of protection from lethal challenge and SN antibody levels are lacking. This study supports the hypothesis that SNA are

critical to protection from challenge.

We may never fully understand why rabies virus has the ability to infect a wide range of mammals with varying incubation periods, varying species susceptibility, and varying virus shedding. These and other factors seem to allow the virus to maintain itself in low level enzootic situations, cyclical epizootic periods, and explosive outbreaks with waves of disease extending from epizootic centers.

Knowledge of the resistance of raccoons to skunk virus and understanding the role of SNA helps in the interpretation of the role of the raccoon in the maintenance and transmission of rabies in skunk enzootic areas.

The most important observations in this study are the resistance of raccoons to skunk virus, the presence of asymptomatic infection as documented by a measurable immune response, and survival of some animals following a lethal challenge. It appears that raccoons require large amounts of skunk virus in order to become infected and that the development of SNA in raccoons exposed to the skunk virus appears to be related to the dose of the inoculum. There is strong correlation between the development of SNA and protection from lethal challenge.

Because the pathogenesis of rabies is so complicated, understanding even a small fraction of the disease in raccoons helps in the understanding of the role of the species in the epizootiology of rabies in the central United States.

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SUMMARY AND CONCLUSIONS

Rabies continues to spread and grow in the world (Beran and Crowley, 1983) and the picture of rabies in the United States has changed dramatically in the last 40 years (Pacer et al., 1985). With the decrease in dog rabies, the major problem in North America is wildlife rabies. Raccoons have emerged as one of several species which are involved in this growing problem. The occurrence of two epizootics of raccoon rabies in the eastern United States (Jenkins et al., 1988), has heightened public health aspects and awareness of the importance of understanding the disease in raccoons. Newer diagnostic methods have also allowed better recognition of the epidemiology of the disease in any host.

The problems of spreading wildlife rabies is not limited to the United States. In Europe, domestic animals rabies has also been reduced dramatically and rabies in wildlife species is expanding. Red fox rabies is found in western Europe and has been found to spread at a rate of 30-60 kilometers per year (Murray, 1987). This expanding epidemic wave has only been temporarily slowed by natural obstructions such as rivers and mountains. Many countries and health organizations are committed to eliminating canine rabies from developing countries throughout the world. As canine rabies is reduced, it is likely that the importance of wildlife reservoirs will increase as has happened in the United States and elsewhere.

The geographic and species specific single reservoir association of rabies and animals is well recognized (Smith et al., 1986). The reason for the association of

particular strains of rabies variants to a particular wildlife species in geographic areas is unknown. Ecologic isolation preventing intra-species transmission does not appear to be a factor. Population studies have shown that as many skunks live in areas without skunk rabies as live in the central United States where skunk rabies is enzootic (Parker, 1975). Species susceptibility to different host adapted viruses appear to be the main factor for lack of inter-species transmission (Sikes, 1962; Parker and Wilsnack, 1966).

Raccoon rabies is not currently a problem in the central United States. However, with the threatened expansion of enzootic raccoon rabies, studies on raccoon rabies in the midwest are especially important in order to understand the current epidemiology of the disease in this species should a raccoon variant ever become established here. In other areas of the country, enzootic raccoon and skunk rabies areas are beginning to overlap (Smith et al., 1986), increasing the epidemiologic complexity of the disease. In addition, raccoon density in the midwest is high, raccoons are highly intelligent and live in close association with people in both rural and urban settings (Hoffman and Gottschang, 1977). In a 1971 study in Florida, there were 5 person/raccoon encounters per 100,000 people yearly (Bigler et al., 1983). In Illinois, exposure potential to rabid raccoons was much greater than to any other species (Schnurrenberger et al., 1969), and in Florida during the period of 1963-1972, 65% of reported human exposures were from contact with raccoons (Bigler et al., 1973).

People are also more likely to encourage the survival of the semi-domesticated raccoon than the skunk. Raccoons are less likely to display the

furious form of rabies (Kappus et al., 1970), yet they often scratch and bite people who approach these animals thinking that raccoons are tame. Public health implications of such contacts are obvious.

There are three basic types of information available on rabies in wildlife. These include reports of confirmed cases from public health and diagnostic laboratories, serologic surveys, and experimental studies. Each provide a different perspective in attempting to understand the epidemiology of the disease in the wild, but all three have drawbacks.

The most common method of determining prevalence of rabies is with data from dead animals submitted to laboratories for rabies examination. These data provide a valuable overall picture of rabies, but there are problems with interpretation of these data. The unreliable nature of public health surveillance data due to differences in submission and reporting policies by states have been documented (Gremillion-Smith and Woolf, 1988). These summaries are often biased towards diseased animals (Woolf and Gremillion-Smith, 1986), and are not reliable indicators of the true prevalence of the disease among wildlife populations (Verts and Storm, 1966). Data from this thesis expand these findings.

Numerous serologic studies of rabies in wildlife have been performed, and there are questions about the validity and interpretation of these data; especially studies with a significant percentage of positive animals with low level serum neutralizing antibodies (SNA). This serologic survey confirms the presence of SNA by both the RFFIT and MSN tests and the results of the IFA test lend evidence that animals with low level serum neutralizing antibody titer have

rabies-specific immunoglobulin G. These data demonstrate that exclusion of all animals with low titer from serologic surveys as has been done in the past is not entirely appropriate.

There have been several experimental studies on the pathogenesis of different rabies variants in different species. Only recently has the raccoon emerged as a wildlife host and there is an incomplete understanding of the role raccoons play in the transmission and maintenance of rabies in wildlife in areas with and without enzootic raccoon rabies.

This study demonstrates that raccoons are relatively resistant to the skunk virus, and that they can survive for extended periods of time following significant challenge. Survival of raccoons challenged with the skunk virus indicates that wider exposure of raccoons to rabid skunks is possible. There is also an indication of the presence of asymptomatic infection as demonstrated by measurable SNA in animals exposed to the virus. Correlation of SNA and protection from challenge is also demonstrated.

It is hopeful that this study has provided some information about the basic dynamics of wildlife rabies. However, it is difficult to draw conclusions about such a complex disease from a small study in two counties in Iowa. If anything, the results emphasize that there are many unanswered questions about wildlife rabies. It is certain that many more questions will arise. Further work in the following areas should be considered as rabies control efforts focus on wildlife reservoirs.

The occurrence of subclinical or abortive rabies needs further study. A

definitive answer to the question of latency of rabies in raccoons also evades us. If inapparent infection exists, it needs to be determined if such animals are a source of secondary transmission and present a source of infection to people and other animals. Although unexpected, it needs to be determined if such animals would have eventually developed fatal clinical disease, and if the distribution of the virus in exposed raccoons is such that it ever reaches the salivary glands.

The overall picture of raccoon susceptibility to the rabies virus is uncertain. This is especially true with the skunk virus. More work in larger numbers of raccoons with different challenge levels are needed before definitive conclusions can be drawn. In addition, experimental studies in skunks with the raccoon adapted virus are needed as the host-associated enzootic areas of these two species begin to overlap.

Virus isolation and characterization of rabies viruses isolated from raccoons found rabid in Iowa are needed to determine if the viruses are skunk, bat, or other variants. Characterization of such isolates by monoclonal antibodies could help determine if the skunk virus variant is the causative agent as suspected. Rabies virus transmission from bats to other species is rare (Tinline, 1988), but should be ruled out.

The occurrence of inapparent infection in species other than the raccoon needs to be determined. As with raccoons, exposure of other wildlife species to rabies virus is unknown, but may be wider than previously believed. In the future, new wildlife species may emerge as primary hosts as the raccoon did 40 years ago. Mechanisms for the adaptation of variants to the primary host should

be investigated.

The mechanism of raccoon survival needs to be determined. It appears that rabies is not as fatal as once believed. Determination of survival mechanisms in animals may be helpful in treating people exposed to rabies. Current hope of recovery in people who develop clinical signs of rabies is infinitely small (Hattwick et al., 1972).

The possible non-specific factors in raccoons sera that have virus-neutralizing ability need further evaluation. This type of information will be critical in understanding serologic based studies. Ongoing work in this laboratory, and reports in the literature indicate that the potential problem of non-specific neutralization is not limited to the raccoon. Further work with the IFA and MSN tests in animals with low level antibodies will also be helpful.

It is uncertain if naturally occurring rabies-specific SNA, or if non-specific factors provide protection from lethal challenge. Continued trapping of raccoons with the hope of finding sufficient numbers of animals with these virus neutralizing factors for experimental evaluation may provide one means of determining the source and protective nature of these factors.

Much work needs to be done before we can make conclusive statements about the true prevalence of rabies in any wildlife species, or the exact role of each species in the maintenance and transmission of the disease.

Rabies is a successful disease. It has been around for millennia; its epidemiology which earlier seemed so simple is actually complex. The virus seems to adapt to attempts at eliminating it as evidenced by the expansion of the

disease in wildlife as rabies in domestic animals was brought under control. The expansion of rabies into wildlife reservoirs and the public health consequences of encounters of people and wildlife necessitate ongoing sylvatic rabies research. If rabies is to be eradicated from the world, an understanding of the epidemiology of the disease is required. Knowledge of the relationship between the virus and its hosts is essential if wildlife rabies control programs are to be expanded.

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ACKNOWLEDGMENTS

I sincerely thank everyone who provided encouragement and support as this project developed. As my major professor, Dr. George Beran has provided guidance and advice throughout my studies. Comments and advice from committee members Drs. Tom Bunn, Howard Hill and Lyle Miller were especially useful. The assistance of Dr. Bunn, Ms. Dee Murphy, Ms. Lea Ann Middle, and Mr. David Halverson in the laboratory was invaluable and greatly appreciated. Special thanks go to Dr. Don Randall, Deputy Director, Veterinary Biologics Field Operations, for giving me the opportunity to complete this thesis. I especially thank my wife Andrea for her infinite love and support.