

Investigation of the Role of Healthy Dogs as Potential Carriers of Rabies Virus

YONG-ZHEN ZHANG,¹ ZHEN F. FU,² DING-MING WANG,³ JING-ZHU ZHOU,³
ZHAO-XIAO WANG,³ TAI-FU LV,³ CHENG-LONG XIONG,¹ YANG ZOU,¹
WEN-RONG YAO,¹ MING-HUI LI,¹ GUAN-MU DONG,⁴ GE-LIN XU,⁵
MICHAEL NIEZGODA,⁶ IVAN V. KUZMIN,⁶ and CHARLES E. RUPPRECHT⁶

ABSTRACT

To investigate whether healthy animals are potential carriers of rabies virus in China, 153 domestic dogs were collected from a rabies enzootic area, Anlong county in Guizhou Province, and monitored for 6 months. Initially, findings of rabies virus antigen in the saliva of 15 dogs by an enzyme-linked immunosorbent assay (ELISA) test suggested they might be carriers. These 15 dogs were kept under observation for 6 months. None of the dogs showed any clinical signs of rabies during the observation period. Moreover, using the ELISA test alone, detection of rabies virus antigen in saliva of some animals was not consistent during the observation period. However, none of the saliva samples collected either at the time of acquisition or during the observation period was found to be positive for rabies virus RNA by reverse transcriptase-polymerase chain reaction (RT-PCR). Furthermore, neither viral antigen nor viral RNA was detected in the brain samples collected at the time of euthanasia. These results do not provide support for the contention that healthy dogs act as carriers in rabies. Caution is urged when preliminary and nondefinitive tests, such as ELISA, are used to infer clinical status related to rabies. **Key Words:** Domestic dogs—Rabies—Healthy carrier—China—Zoonosis—Virus.

INTRODUCTION

RABIES IS ONE OF THE MOST important and widespread zoonotic diseases. The disease is caused by RNA viruses in the family Rhabdoviridae, genus *Lyssavirus*. Natural productive infections in all mammalian species studied generally cause an acute, progressive fatal encephalitis (Warrell and Warrell 2004). Most previous studies have shown that experimentally or naturally infected dogs do not survive

more than approximately 9 days after the onset of clinical signs of rabies (Vaughn et al. 1965, Tepsumethanon et al. 2004). However, rabies virus-specific antibodies detected in apparently healthy mongooses, skunks, raccoons, foxes, hyenas, jackals, fruit bats, vampire bats, and insectivorous bats suggest that these animals have been infected and somehow survived to be persistent carriers (Aghomo et al. 1990, East et al. 2001, Everard et al. 1981, Lord et al. 1975, Mebatsion et al. 1992, Rosatte and Gunson

¹Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, China.

²Department of Pathology, College of Veterinary Medicine, University of Georgia, Athens, Georgia.

³Guizhou Center for Disease Control and Prevention, Guiyang, China.

⁴National Institute of the Control of Pharmaceutical and Biological Products, Beijing, China.

⁵Wuhan Institute of Biological Products, Wuhan, China.

⁶Rabies Program, Division of Viral and Rickettsial Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia.

1984, Trimarchi and Debbie 1977). For example, East et al. (2001) suggested that 37% of hyenas were seropositive for rabies virus antigens, and almost half of the seropositive hyenas were positive for rabies virus RNA by reverse-transcriptase-polymerase chain reaction (RT-PCR) in their saliva. However, no rabies virus was isolated from any of the saliva samples (East et al. 2001). Rabies virus-specific antibodies have also been reported in domestic dogs in Ethiopia (Ogunkoya et al. 1990). In exceptional cases, virus excretion in the saliva of experimentally infected dogs may occur for as long as 14 days before detectable clinical signs of rabies (Fekadu et al. 1982). An example is a dog that shed rabies virus intermittently in its saliva for up to 305 days after supposed recovery from experimental infection with an Ethiopian strain of rabies virus (Fekadu et al. 1981). In India, a dog was reported to have no detectable antibody in saliva but excreted rabies virus intermittently in its saliva for over 30 months, and rabies virus has also been reported as isolated (Veeraraghavan 1970). Rabies virus was reported as isolated from apparently healthy dogs in other countries as well (Aghomo and Rupprecht 1990). Such suggestions that apparently healthy dogs may shed rabies virus in their saliva over long periods of time, and thus might transmit the infection to other animals, including humans, are a major concern.

Recently, human rabies cases have been increasing steadily in China, with at least 2651 and 2548 cases in 2004 and 2005, respectively. In 2006, a total of 3273 human cases were reported, an almost 30% increase compared with those reported in 2005. The number of dogs (more than 70 million) and low coverage of vaccination (less than 3%) contributed to the increasing human rabies cases (Tang et al. 2005, Zhang et al. 2005, 2006a,b). Rabies virus antigen has been reported in supposedly healthy domestic dogs in endemic areas (Du et al. 1992, Li et al. 2004, Lu et al. 2006). As many as 4% of the sampled brains were reported as positive for rabies virus antigen (Xu et al. 1999). Furthermore, Yu et al. (2002) also reported that rabies virus antigen was detected in saliva samples in more than 4% of healthy domestic dogs. In addition, rabies virus was reported as isolated from the brain of healthy dogs in endemic

areas (Xu et al. 1999, Zhang et al. 2006a). Taken together, these studies have been cited to suggest that healthy dogs may be carriers of rabies virus. If confirmed, such observations would have important public health implications for rabies epidemiology in general, and specifically for practical human postexposure prophylaxis. Currently, after human exposure, the suspected dog(s) may be observed for at least 10 days. If the animal remains healthy, no human prophylaxis is necessary. In contrast, if healthy dogs suspected of carrying rabies virus existed widely, the historical rules for domestic animal observation become questionable as guidelines for effective public health decision making following a dog bite. Moreover, if there is a belief that healthy dogs may be carrying rabies, many healthy dogs will be innocently killed under the guise of rabies control in China. With the increase of the pet population and the overall low coverage of rabies vaccination in China (Tang et al. 2005, Zhang et al. 2005, 2006a,b), it is imperative to determine whether the dogs are a risk (through biting) to humans and provide the opportunity for rabies virus transmission. In the present study, our objective was to examine the issue of whether healthy dogs are potential carriers of rabies virus by trapping dogs in endemic areas of China, and monitoring development of neurological disease and virus excretion in saliva for at least 6 months.

MATERIALS AND METHODS

Animals

To investigate the concept of animal carriers for rabies, apparently healthy domestic dogs were trapped in a rural area of Anlong county, Guizhou Province, during 2005. Saliva was collected from each dog and tested for the presence of rabies virus antigen by a commercial enzyme-linked immunosorbent assay (ELISA) test. All suspect dogs, positive for rabies virus antigens in saliva, were kept individually in a dog run for 6 months. Saliva was collected at an interval of every 10 days during the observation period. Sera were also collected from each dog after the first and last collections of

saliva. The dogs were euthanized, and the sera, cerebrospinal fluid, and brains collected at the end of the study. All samples were frozen immediately in liquid nitrogen and transported to the laboratory for further analysis.

Screening for rabies virus antigens

Rabies virus antigen (N) in saliva was detected using commercial ELISA kits according to the manufacturer's instructions (Wuhan Institute of Biological Products, Wuhan, China). Briefly, the microtiter plate was coated with mixed antirabies virus N monoclonal antibodies, incubated with the test material, and the antigen-antibody complex detected with peroxidase-conjugated antirabies virus N protein monoclonal antibodies as described (Xu et al. 1999, Xu et al. 2007). Rabies virus antigen in dog brain was detected by using a direct rapid immunohistochemical test (dRIT) with antirabies virus N monoclonal antibody, as described below (Lembo et al. 2006).

dRIT procedure

Touch impressions of fresh dog brain tissue (brain stem and cerebellum) were made on 2-well white Teflon-coated microscopic slides, with the same protocol as used for the direct fluorescent antibody test, as described (<http://www.cdc.gov/ncidod/dvrd/rabies/>). Slides were air-dried for 5–10 minutes and then immersed in 10% buffered formalin for 10 minutes at ambient temperature. The slides were dip-rinsed in phosphate-buffered saline (PBS) with 1% Tween 80 (TPBS) and immersed in 3% hydrogen peroxide for 10 minutes. After a dip-rinse in TPBS, the slides were incubated in a humidity chamber (e.g., a plastic top to a 96-well plate or another simple cover on a moistened paper towel on the laboratory bench top) with biotinylated antirabies virus monoclonal antibodies directed against the virus N protein for 10 minutes at ambient temperature. After a dip-rinse in TPBS, slides were incubated with a streptavidin-peroxidase complex for 10 minutes at ambient temperature. Slides were dipped in TPBS and incubated with the peroxidase substrate 3-amino-9-ethylcarbazole in dimethyl-formamide with acetate buffer as diluent for 10 minutes at ambient temperature.

Slides were dipped in distilled water and counterstained with Gills hematoxylin (diluted 1:1 with distilled water) for 2 minutes at ambient temperature and then in distilled water to remove excess stain. Slides were mounted with a water-soluble mounting medium. Finally, slides were covered with a coverslip and read using a light microscope at $\times 200$ to $\times 400$ total magnification.

Screening for rabies virus RNA

RNA was extracted from dog saliva with a TRIzol reagent according to the manufacturer's instructions (Invitrogen, Beijing, China). This RNA was used to amplify fragments of rabies virus N gene by nested RT-PCR, as described previously (Zhang et al., 2006a). Primers RHN1 and RNHS3 (Ito et al. 2001) were used for the first amplification and primers N53 and N55 (Bourhy et al. 1999) were used for the nested amplification. Additionally, primers N550F (ATGTGYGCTAAYTGGAGYAC, sense, nucleotides [nts] 647–666 according to the SAD B-19 genome, GenBank accession no. M31046) and 304 (TTGACGAAGATCTTGCTCAT, anti-sense, nts 1514–1533 according to SAD B-19 genome) were used for the first amplification, whereas primers 1066 (GAGAGAAGATTCTTCAGGGA, sense, nts 1136–1155 according to the SAD B-19 genome) and 105 (TTCTTATGAGTCACTCGAATATGTCTTGTGTTAG, anti-sense, nts 1394–1426 according to the SAD B-19 genome) were used in the nested amplification. The reactions were performed as described elsewhere (Hughes et al. 2006). The procedure was performed in the Chinese Center for Disease Control and Prevention (CDC) by investigators from the U.S. CDC. The procedure and primer sets are used routinely in the United States for rabies diagnosis from samples collected worldwide.

Rabies virus antibody detection in sera and cerebrospinal fluid

Rabies virus-specific virus-neutralizing antibody titers were determined by the rapid fluorescent focus inhibition test (RFFIT) as described previously (Smith et al. 1973). The World Health Organization (WHO) international reference serum was included to deter-

mine antibody titers in international units (IU)/mL. Samples with titers ≥ 0.5 IU/mL were considered positive.

RESULTS

The selected study site, Anlong county in Guizhou Province, is one of the major rabies endemic areas in China. Although no human rabies cases had been reported from 1994 to 2003 in Anlong, 44 and 27 human rabies cases occurred in 2004 and 2005, respectively (Zhang et al. 2006b). Among the total of 71 human rabies cases, 68 (96%) were attributed to dog bites (Zhang et al. 2006b). Anecdotal evidence indicated that people bitten by apparently healthy dogs succumbed to rabies, suggesting that healthy dogs were potential rabies carriers (Wu 1982). To investigate if healthy local dogs were apparent rabies carriers and an acute problem for rabies control measures, a total of 153 domestic dogs were trapped in Anlong county. None of these dogs was immunized with rabies vaccine. All were apparently healthy at the time of trapping (Fig. 1a). Saliva was collected from each dog (Fig. 1b), and rabies virus antigen was detected in the saliva of 15 of the 153 dogs examined. Based upon these preliminary results, ELISA-positive samples were detected in 15 dogs. Subsequently, these 15 dogs were monitored for 6 months by keeping each dog confined in a separate area. During the observation period, all dogs remained healthy and no apparent clinical signs suggestive of rabies were observed. Rabies virus antigen was detected intermittently using the ELISA kit (Table 1).

In an attempt to confirm these preliminary ELISA results, all saliva samples were tested for rabies virus RNA by RT-PCR. No positive samples were detected. At the end of the observation period, all 15 dogs were euthanized. Their brains were harvested and used for detection of rabies virus antigen by the dRIT assay with the antirabies virus N monoclonal antibodies. Mouse brain infected with the ERA strain was included as a positive control in the field. All brain samples from the dogs were negative for rabies virus N (Fig. 2), although the mouse brain infected with the ERA virus demonstrated positive staining (data not shown).

As summarized in Table 2, virus-neutralizing antibodies ≥ 0.5 IU were detected in 2 serum samples collected at the beginning of the experiment. One sample showed antibody titers closer to the 0.5 IU. However, antibody titers declined at the second collection, indicating that no virus infection occurred during the observation period. Furthermore, no virus-neutralizing antibodies were detected in any of the cerebrospinal fluid samples (data not shown).



FIG. 1. Dogs were trapped in endemic rabies foci in Anlong county (a), and the saliva was collected from an ELISA-positive dog before being euthanized (b).

TABLE 1. RABIES VIRUS ANTIGEN DETECTED IN CANINE SALIVA BY ELISA TESTING

Dogs	When trapped	Observation period (at an interval of every 10 days)																
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
AL2	+	+	-	-	-	-	+	-	-	+	-	-	+	-	+	-	-	-
AL3	+	+	-	-	-	-	-	+	+	+	-	-	-	-	-	+	-	+
AL5	+	-	-	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-
AL6	+	-	-	+	-	-	-	+	-	-	-	-	-	+	-	-	-	-
AL9	+	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-
AL21	+	-	+	+	-	-	-	+	-	-	+	-	-	+	-	-	+	-
AL23	+	+	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-	+
AL45	+	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	+	-
AL54	+	-	-	-	-	+	-	+	-	-	-	+	-	-	+	+	-	-
AL55	+	+	-	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-
AL81	+	-	-	-	-	-	+	-	+	-	+	+	-	-	-	+	-	-
AL82	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-
AL93	+	+	-	-	+	-	-	-	-	-	+	-	-	-	+	-	-	+
AL98	+	+	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-
AL150	+	-	-	+	-	-	-	-	+	-	-	-	+	-	-	+	-	-

DISCUSSION

If healthy dogs were potential rabies carriers, this would increase rabies transmission not only among the dog population, but also to humans. People bitten by healthy dogs may not seek postexposure prophylaxis and thus may develop rabies. On the other hand, more healthy dogs are killed innocently because of the perception that healthy dogs are carriers. In the present study, we investigated if healthy dogs are rabies carriers in one of the rabies endemic areas in China. Although rabies virus antigen was detected presumably in the saliva of 15 dogs as identified by the ELISA test initially, none of them could be confirmed as carriers of rabies virus by RT-PCR. Furthermore,

none of these dogs developed clinical signs of rabies during the 6-month observation period, and no rabies virus antigen or RNA was detected in the brain tissue of these dogs when euthanized at the end of the observation period. Thus, these results taken together do not provide any objective substantive support for the contention that healthy dogs are rabies carriers in China.

Only low levels of virus-specific neutralizing antibodies were detected in 2 of the 15 samples collected at the beginning of the experiment. During the observation period, no antibody increase was detected in any of the dogs, indicating that no active infection was present in these dogs. Furthermore, no neutralizing antibodies were detected in the cerebrospinal fluid

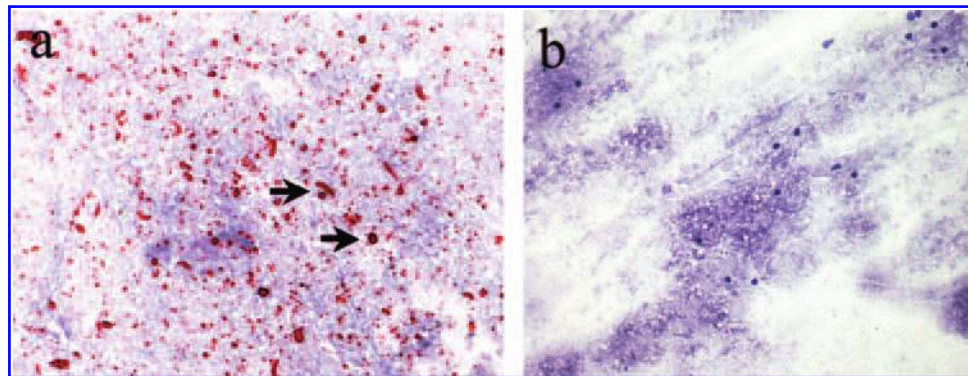


FIG. 2. Detection of rabies virus antigen using a direct rapid immunohistochemical test (dRIT). Positive touch impressions were fixed briefly in formalin and stained with antirabies virus nucleoprotein antibodies as described (Lembo et al. 2006). (a) Arrows and red staining inclusions indicate the identification of rabies virus nucleoprotein in the mouse brain infected with the ERA vaccine strain. (b) A dog brain shows no viral antigen as described in the Materials and Methods.

TABLE 2. DETECTION OF RABIES VIRUS-NEUTRALIZING ANTIBODIES IN SERA

Samples	Neutralizing antibodies in sera	
	First collection	Last collection
AL2	—	—
AL3	—	—
AL5	—	—
AL6	—	—
AL9	—	—
AL21	≥0.84	≥0.46
AL23	—	—
AL45	—	—
AL54	—	—
AL55	—	—
AL81	—	—
AL82	—	—
AL93	≥0.69	≥0.46
AL98	—	—
AL150	≥0.46	≥0.18

from any of these dogs when euthanized, supporting the belief that no rabies virus infection of the central nervous system was present. This observation is in contrast to other reports in the field, wherein rabies virus antibodies were detected in apparently healthy wildlife (Warrell and Warrell 2004). East and colleagues (2001) found that 37% (37/100) of spotted hyenas were seropositive for rabies virus in the Serengeti National Park, Tanzania. Moreover, Ogunkoya et al. (1990) also reported that rabies virus antibodies were detected in unvaccinated and stray dogs, and even neutralizing antibodies to Mokola and Lagos bat viruses.

Natural infection of rabies virus in animals does not necessarily mean that a "carrier state" is established. Vaughn et al. (1963, 1965) reported that salivary excretion of rabies virus could not be detected in cats and dogs except approximately 3–4 days prior to the onset of illness. Experimentally or naturally infected cats and dogs do not survive more than 9 days after the onset of clinical signs of rabies (Tepsumethanon et al. 2004, Vaughn et al. 1963, 1965). However, Fekadu et al. (1982) reported that the longest duration from the first salivary excretion of rabies virus to death in their study animals was 14 days. Furthermore, one dog even intermittently shed rabies virus in its saliva for up to 305 days after recovery from the experimental infection with an Ethiopian

strain of street virus (Fekadu et al. 1981). Rabies virus antigens have been detected in apparently healthy domestic dogs from endemic areas in China (Du et al. 1992, Li et al. 2004, Lu et al. 2006, Xu et al. 1999). Furthermore, rabies virus has been isolated from such apparently healthy dogs (Xu et al. 1999, Zhang et al. 2006a). Virus isolation was made from brain tissue, not from saliva. It is thus possible that those animals were still in the prodromal period. In the present study, an ELISA test was initially used to detect rabies virus antigens in the brain and the saliva. The ELISA test was developed for testing of rabies virus antigen in brain and has been validated by virus isolation (Xu et al. 1999). However, the use of such a test to detect rabies virus antigen in saliva has not been validated (Yu et al. 2002). The saliva samples that were positive for rabies virus antigen by the ELISA could not be confirmed by RT-PCR, suggesting that the ELISA is not appropriate for reliable detection of rabies virus in saliva. Neither virus antigen nor viral RNA was detected in the brain and saliva of suspect dogs by RT-PCR in our present study, indicating that prior suggestions of a carrier state may suffer from mistaken identity of the offending animal, provoke inappropriate recall bias, or simply represent nonspecific laboratory results. Clearly, such results must be interpreted with caution. In this limited study in an enzootic area, we can provide no corroborative evidence for the existence of healthy carrier dogs in China.

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Address reprint requests to:

Yong-Zhen Zhang

Institute for Communicable Disease Control
and Prevention

Chinese Center for Disease Control
and Prevention

Changping Changping Liuzi 5
Beijing 102206
China

E-mail: yongzhenzhang@sohu.com

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