

# REAL-TIME PCR ANALYSIS OF DOG CEREBROSPINAL FLUID AND SALIVA SAMPLES FOR ANTE-MORTEM DIAGNOSIS OF RABIES

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**Abstract.** The use of a 10-day observation to determine whether a dog is rabid is standard practice. This study was conducted in order to look for evidence of rabies virus in saliva and cerebrospinal fluid (CSF) of suspected live rabid dogs at the time of quarantine by using a SYBR Green real-time RT-PCR based assay for the detection of rabies virus RNA. Saliva and CSF of dogs were collected once on the day of admission for the 10-day quarantine. All test dogs were or became ill and died of rabies within the observation period. Thirteen of 15 dogs (87%) had saliva samples that were positive for rabies RNA. Two dogs with furious rabies had negative saliva samples. Positive CSF samples were found in 4 of 15 dogs (27%) whose saliva samples were positive. The time from sample collection to result was less than 5 hours. Because virus may be absent or present at very low level in both clinical fluids, samples taken for ante-mortem diagnosis cannot definitively rule out rabies.

## INTRODUCTION

Dog is the most common animal infected with rabies. Suspected rabid dogs are usually killed and brains are examined for the virus. Alternatively, the dogs are placed under observation for 10 days. However, making a reliable diagnosis of rabies based on clinical presentation can be difficult to distinguish from encephalitic conditions cause by other viral infections (Niezgoda *et al*, 2002). A biting dog, particularly in a rabies enzootic region, should be killed whenever possible and tested by using fluorescent antibody techniques (Trimarchi and Smith, 2002). This is difficult in Buddhist countries which abhor killing of ani-

mals and prefer having them caged and observed for clinical signs. Laboratory examination in combination with a 10-day observation can be used to derive more precise ante-mortem diagnosis of dog rabies. There is no ideal ante-mortem diagnostic test for rabies but saliva and cerebrospinal fluid (CSF) evaluation may provide a valuable tool for this approach. However, no reports have been published demonstrating the value of intravital laboratory diagnosis of rabies in dogs. This study was aimed to determine whether rabies virus was present in saliva or CSF of biting dogs at the time of quarantine in comparison to brain after death, and to determine the adequacy of using real-time PCR with SYBR Green I for detecting rabies virus in both clinical fluids.

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## MATERIALS AND METHODS

### Clinical specimens

Fifteen dogs suspected of being rabid

admitted to Queen Saovabha Memorial Institute, Bangkok, Thailand were kept in individual cages and observed daily. Specimens of saliva and CSF were collected from each dog once on the day of admission. Cisternal puncture to obtain CSF was done under deep barbiturate anesthesia. Saliva was collected with sponge-tipped applicators and stored as described previously (Kasempimolporn *et al*, 2000). All dogs were ill or showed unusual behavior and finally died within a 10-day-observation period. Clinical signs were observed and recorded by veterinary specialists. Clinical features of dog rabies can be classified as furious and dumb. Furious form is a clinical presentation that consists predominantly of profound agitation and aggression. With the dumb form, aggression may be completely lacking, but paralysis may be paramount. Rabies virus was demonstrated by the fluorescent antibody test (FAT) (Dean *et al*, 1996) of brain tissue from all of dogs.

#### Real-time RT-PCR with SYBR Green I dye

Total RNA was extracted from saliva or CSF specimens using TRIzol® (GIBCO-BRL, Gaithersburg, Md, USA). RT-PCR of 2 µg of extracted RNA was performed in a one-step process using AccessQuick® kit (Promega, Madison, WI, USA). Amplifications were carried out on a thermocycler (MWG-Biotech, Germany), using a denaturation temperature of 94°C, an annealing temperature of 60°C, and an extension temperature of 72°C. Subsequently, the amplified product was subjected to a second-round PCR using the SYBR Green I protocols. The assay was performed with LightCycler DNA Master SYBR Green I kit (Roche Molecular Biochemicals, France), according to the manufacturer's instructions. Five µl of the first-round PCR product were added to 15 µl of master mix. The thermal cycling conditions used were as follows: 95°C for 0 second, 55°C for 10 seconds and 72°C for 13 seconds. A total of 45 cycles were performed and ending with a melting curve analy-

sis for product identification at 95°C for 0 second, 65°C for 10 seconds and 95°C for 0 second. All acquired fluorescence data were analyzed using LightCycler software. In this study, two sets of primers were used to amplify 524 bp (5'-GACATGTCCGGAAGACTGG-3', at position 319-337, and 5'-GTATTGCCTCTCTA GCG GTG-3', at position 823-842) and 123 bp (5'-GTAACACCTCTACAATGGATGC-3', at position 57-78, and 5'-TCAAATCTTTGATGGCA GGGTA-3', at position 158-179) fragments of rabies virus nucleoprotein gene, for the first- and second-round PCR, respectively. To test sensitivity, serial dilutions of known RNA amounts from fixed CVS rabies virus were subjected in parallel to PCR examination.

## RESULTS

Specimens of saliva and CSF were examined for the presence of rabies virus RNA by real-time RT-PCR using SYBR Green I dye as a monitor of specific amplicons. A typical nucleic acid amplification plot and melting curve analysis is shown in Fig 1. The tall sharp peaks indicate the melting temperature of the rabies virus PCR fragment amplified from positive saliva or CSF samples. The short peaks obtained were non-specific products and due to primer-dimer formation. To confirm the amplicon size, the PCR products were visualized by gel electrophoresis. A 123 bp of the rabies virus nucleoprotein gene was observed (data not shown).

Of the 15 proven rabid dogs, eleven (73%) had furious and four (27%) had dumb rabies. Thirteen of them (87%) had saliva samples that were positive for rabies RNA. The remaining two dogs with furious form had no rabies RNA detectable in saliva. Positive CSF samples identified in only 4 of 15 test dogs (27%) whose saliva samples were also positive. Rabies was confirmed in the brain tissue of all 15 dogs by post-mortem examination using FAT (Table 1). The genome detection limit was approximately

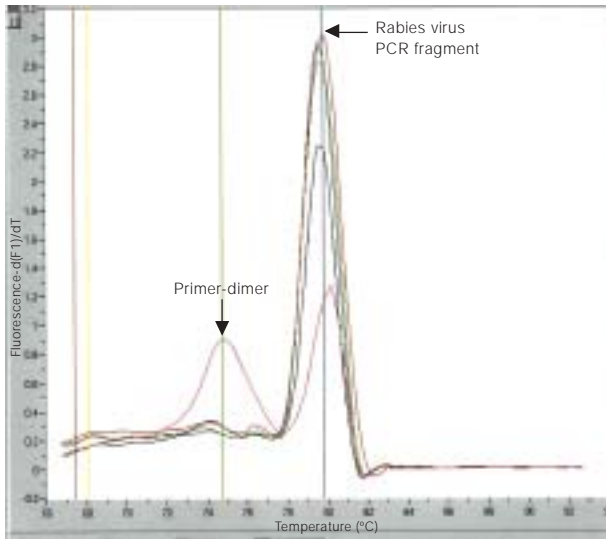


Fig 1–Melting curve analysis of rabies virus PCR fragment and primer-dimers. The melting temperature ( $T_m$ ) for the rabies virus PCR fragment is 79.8°C, and the  $T_m$  for the primer-dimers is less than 75°C. Each curve represents samples tested positive for rabies virus with various quantities.

5 pg when using fixed CVS rabies virus as the source of the starting RNA template (data not shown).

DISCUSSION

The most important factor in transmission of the disease is the presence of virus in saliva. Rabies virus may be excreted in the saliva of dog before clinical signs appear and may lead to infection of an unexpected and untreated bite victim. The particular time of salivary virus excretion before sickness is crucial. Typically, rabid dogs shed virus concomitant with illness or a few days before (Fekadu *et al*, 1982). In naturally infected dogs the rate at which virus is present in the salivary glands ranges from 75-100% (Fekadu and Shaddock, 1984). The clinical presentation of dog rabies often is generalized as being either furious or dumb. Approximately 70% of infected dogs had the furious type and 30% the dumb form (Tepsumethanon *et al*, 2004). A suspected dog should be euthanized and the brain examined

Table 1  
Results of rabies virus detection in saliva and CSF from living rabies suspected dogs.

Dog no.	Clinical type	Saliva	CSF	Brain at death
1	Dumb	+	+	+
2	Furious	+	-	+
3	Furious	+	-	+
4	Furious	-	-	+
5	Furious	+	-	+
6	Furious	+	-	+
7	Dumb	+	+	+
8	Furious	+	-	+
9	Furious	-	-	+
10	Dumb	+	+	+
11	Furious	+	-	+
12	Furious	+	-	+
13	Furious	+	-	+
14	Dumb	+	+	+
15	Furious	+	-	+
Sensitivity		13/15 (87%)	4/15 (27%)	15/15 (100%)

for rabies virus. The most difficult decisions arise when the offending dog cannot be sacrificed promptly because religious scruples against killing prevail. Alternatively, a dog can be quarantined and observed for a 10-day period or until a definitive diagnosis can be obtained. If the dog remains entirely well for 10 days, it can be assumed that the dog did not have rabies in its saliva at the time of the bite (Mann, 1985).

Recently developed laboratory techniques permit the diagnosis of rabies in animals in which rabies virus is suspected as the cause of clinical illness. Ante-mortem diagnosis looks for evidence of the virus in saliva and CSF. The sensitivity of all techniques may vary according to the stage of clinical illness and the intermittent nature of virus shedding (Niezgoda *et al*, 2002). In live animal cases the speed of diagnosis is of paramount importance. A rapid diagnosis could save time and improve the safety of veterinary staff. A purported ante-mortem latex agglutination test for rabies virus antigen has been developed for evaluation of dog saliva (Kasempimolporn *et al*, 2000). The technique gives good results and is comparatively simpler to perform than the standard FAT which needs brain tissue. A confirming method applicable in a living suspected dog would, nevertheless, be desirable. More sensitive molecular techniques such as conventional- and real-time PCR assays have produced satisfactory results for detection of rabies virus RNA from brain tissue and saliva (Tordo *et al*, 1995, Black *et al*, 2002; Nagaraj *et al*, 2006). For real-time PCR, the simplest and most economical format is the one employing double-strand DNA-specific SYBR Green dye. The use of real-time SYBR Green PCR methodology offers several potential advantages over conventional PCR assays, namely more rapid results, no time-consuming post-PCR analyses. In addition, when standards of known amounts are used, quantitative results can be obtained.

In this study, approximately two-thirds of 15 dogs with clinical signs of rabies had the furious form and the remaining dogs developed dumb rabies. We could not demonstrate the virus in saliva of two test dogs with furious type. This could be explained by the fact that the load of infective virus in saliva is lower than that in brain and salivary glands. The intermittent nature of virus shedding in the saliva of an infected dog might also be one of the possible explanations. There may well have been that all dog saliva samples in this study that would have been positive if tested more than once. Therefore, the testing of serial saliva samples taken at different time intervals is necessary. We found that rabid dogs, on rare occasions, have virus in the salivary glands and brain at death but yet have no virus excreted in the saliva during life (unpublished data). This may explain why not every rabid dog bite results in clinical rabies. The fact that rabies virus could only be found in 4 of 15 rabid dog CSF samples is surprising since rabies is an encephalitis. Our findings confirm the previous reports that found virus infrequently in human CSF of rabies patients (Crepin *et al*, 1998; Wacharapluesadee and Hemachudha, 2001).

The SYBR Green real-time PCR assay has potential for the qualitative detection of rabies virus in saliva and CSF of suspected live rabid dogs. In this study, it would seem that saliva appears to be a better source of rabies virus than CSF in the ante-mortem diagnosis. The collection of saliva is much easier than CSF. However, nested PCR is required in all cases to compensate for the often extremely limited amount of RNA in ante-mortem samples (Kasempimolporn *et al*, 2007). The sensitivity of the real-time PCR assay was comparable to those achieved using conventional technique (data not shown). The time from sample collection to result was less than 5 hours. We conclude that a rapid and sensitive test for rabies virus that can be performed

on a living rabies suspected dog can allow immediate diagnosis. It can help the veterinarian to decide whether prompt sacrificing of the dog is indicated. Moreover, it can support a decision to institute immediate post-exposure treatment of exposed humans. A negative test, on the other hand, does not exclude rabies.

## REFERENCES

- Black EM, Lowings JP, Smith J, Heaton PR, Mc Elhinney LM. A rapid RT-PCR method to differentiate six established genotypes of rabies and rabies related viruses using TaqMan technology. *J Virol Methods* 2002; 105: 25-35.
- Crepin P, Audry L, Rotivel Y, Gacoin A, Caroff C, Bourhy H. Intravital diagnosis of human rabies by PCR using saliva and cerebrospinal fluid. *J Clin Microbiol* 1998; 36: 1117-21.
- Dean DJ, Abelseth MK, Atanasiu P. The fluorescent antibody test. In: Meslin FX, Kaplan MM, Koprowski H, eds. Laboratory techniques in rabies. Geneva: World Health Organization, 1996: 88-95.
- Fekadu M, Shaddock JH, Bear GM. Excretion of rabies virus in the saliva of dogs. *J Infect Dis* 1982; 145: 715-9.
- Fekadu M, Shaddock JH. Peripheral distribution of virus in dogs inoculated with two strains of rabies virus. *Am J Vet Res* 1984; 45: 724-9.
- Kasempimolporn S, Saengseesom W, Lumlertdacha B, Sitprija V. Detection of rabies virus antigen in dog saliva using a latex agglutination test. *J Clin Microbiol* 2000; 38: 3098-9.
- Kasempimolporn S, Sichanasai B, Saengseesom W, Puempumpanich S, Chatraporn S, Sitprija V. Prevalence of rabies virus infection and rabies antibody in stray dogs: a survey in Bangkok, Thailand. *Prev Vet Med* 2007; 78: 325-32.
- Mann J. Immunization against rabies, pre-and post-exposure. In: Winkler WG, ed. Rabies concepts for medical professionals. Florida: Merieux Institute, 1985: 51-8.
- Nagaraj T, Vasanth JP, Desai A, Kamat A, Madhusudana SN, Ravi V. Ante-mortem diagnosis of human rabies using saliva samples: comparison of real time and conventional RT-PCR techniques. *J Clin Virol* 2006; 36: 17-23.
- Niezgoda M, Hanlon CA, Rupprecht CE. Animal rabies. In: Jackson AC, Wunner WH, eds. Rabies. San Diego: Academic Press, 2002: 163-218.
- Tepsumethanon V, Lumlertdacha B, Mitmoonpitak C, Sitprija V, Meslin FX, Wilde H. Survival of naturally infected rabid dogs and cats. *Clin Infect Dis* 2004; 39: 278-80.
- Tordo N, Bourhy H, Sacramento D. Polymerase chain reaction technology for rabies virus. In: Clewley JP, ed. The polymerase chain reaction (PCR) for human viral diagnosis. Boca Raton: CRC Press, 1995: 125-45.
- Trimarchi CV, Smith JS. Diagnostic evaluation. In: Jackson AC, Wunner WH, eds. Rabies. San Diego: Academic Press, 2002: 308-49.
- Wacharapluesadee S, Hemachudha T. Nucleic acid sequence based amplification in the rapid diagnosis of rabies. *Lancet* 2001; 358: 892-3.