JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2000, p. 3098–3099 Vol. 38, No. 8
0095-1137/00/$04.00+0

Detection of Rabies Virus Antigen in Dog Saliva Using a Latex Agglutination Test

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Received 7 October 1999/Returned for modification 26 February 2000/Accepted 29 May 2000

Dog bites are responsible for more than 90% of human rabies deaths in Asia. We developed a simple and inexpensive test based on latex agglutination (LA) for rabies virus antigen detection in dog saliva. Rabies virus antigen could be detected by agglutination on a glass slide using latex particles coated with gamma globulin. By evaluation of paired saliva-brain specimens from 238 dogs, the LA test using saliva was 99% specific and 95% sensitive compared to the fluorescent antibody test (FAT) on brain smears. The advantages of the LA test over the standard FAT are that it is comparatively simple and there is no need to kill the animal before examination.

Rapid and accurate laboratory diagnosis of rabies is essential for timely administration of postexposure prophylaxis. Reliable reports may save a patient unnecessary psychological trauma and financial burdens. The "gold standard" in rabies diagnosis is the fluorescent-antibody test (FAT) on brain tissue. The mouse inoculation test (MIT) has traditionally served as a quality control method for the FAT but has serious flaws. The FAT has a sensitivity of 99.78% when carried out in a laboratory by experienced workers (16). However, the FAT requires expensive reagents and instruments, well-trained technicians, and necropsy material. The sensitivity of the test is substantially reduced once brain specimens start to decompose. This can pose a problem in tropical countries, where transportation of specimens to a regional diagnostic laboratory often entails delay. In cases of rabies suspected in humans antemortem diagnosis may be achieved by several techniques. Isolation of the virus from the patients' saliva, tears, cerebrospinal fluid, or urine by mouse inoculation is possible but requires at least 1 week and is unreliable. The presence of rabies virus antigen, as demonstrated by a positive FAT result, in salivary or corneal smears (11, 18) and in nuchal skin biopsy samples (2) is considered diagnostic. These procedures are less invasive than brain biopsy. Excluding brain biopsy, immunohistochemical examination of a sample obtained by skin biopsy remains the most sensitive test, but results depend on the stage of disease at the time of the biopsy (1). Biopsy during the late stage yields more positive results. More-sensitive but -sophisticated and -expensive techniques such as PCR have produced satisfactory results for detection of rabies virus antigen in brain (9) and in cerebrospinal fluid and saliva specimens (3). Enzyme immunoassay (13) and dot hybridization (4) have also been adapted to detect rabies virus antigen in brain and salivary glands. There is as yet no assay that has been used as a replacement for FAT and/or MIT for routine diagnosis. The reasons for this are technical and logistic. The above-mentioned methods showed no significant advantage over FAT and, with the exception of PCR, could not solve the problem created by decomposed specimens.

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The present study involved the development of a simple and inexpensive test for diagnosis of rabies based on latex agglutination (LA) using dog saliva. We also evaluated the sensitivity and specificity of the LA test by comparing it with the conventional FAT on brain smears.

Dog brains were collected from carcasses submitted to the rabies diagnostic unit at Queen Saovabha Memorial Institute. Brain stem impressions were examined by FAT (16). Samples from all brains with negative FAT results were submitted for MIT. Saliva was collected from the same samples by immersing a sponge swab into 2 ml of phosphate-buffered saline (PBS) and then swabbing the anterior surface of the tongue and the cheek mucosa of the dog for 15 to 20 s. The swab was immersed in PBS and kept at 4°C. Each saliva sample was centrifuged at 12,500 × *g* for 20 min prior to examination by LA.

Antiserum was produced by immunizing horses with a purified Vero cell rabies virus vaccine (Institute Merieux, Lyon, France). The horses were given a series of vaccine injections in increasing doses. All the injections were given subcutaneously into the lateral aspect of the neck. The immunization period lasted for 105 days, and the first bleeding was carried out 14 days later (12). Gamma globulin (IgG) was fractionated from serum by DEAE-cellulose column chromatography as previously described (10). Latex particles from two manufacturers were used: polystyrene latex beads (diameter, 0.50 μm; Takeda Pharmaceutical Co., Osaka, Japan) and blue polystyrene latex beads (diameter, 0.25 μm; Sigma, St. Louis, Mo.). The sensitized latex beads were prepared as follows. A 1% suspension of latex beads in PBS (pH 7.2) was mixed with an equal volume of IgG (3 mg/ml) and shaken at room temperature for 2 h. The mixture was then washed twice with PBS by centrifugation at 9,650 × *g* for 5 min. The pellet was suspended in PBS containing 1% bovine serum albumin and kept overnight at 4°C. After being washed twice with PBS, the sensitized latex beads were resuspended in latex diluent (PBS with 1% bovine serum albumin and 0.005% polyvinylpyroridone [wt/vol]) at a concentration of 0.4% and kept at 4°C until used.

Two 25-μl aliquots of coated latex beads were layered on a double-concave slide, one with 25 μl of the saliva to be tested and one with 25 μl of PBS as a negative control. After gentle mixing with vortex, agglutination was judged macroscopically against a dark background.

Preliminary experiments on LA alone were done to discover

problems and to select the most desirable latex particle size. Two types of commercial latex beads were coated with anti-rabies virus IgG at various concentrations. The test was initially performed with rabies virus from the culture supernatant of infected BHK-21 cells and with some saliva samples from dogs which were previously known to be positive or negative for rabies virus infection. Particle size and IgG concentration affected the sensitivity. The most desirable particle size was 0.25 μm (blue latex) because particles of this size agglutinated the virus specifically. When the higher IgG concentration was used, a higher sensitivity was obtained. With the sensitized blue latex beads coated with 3 mg of IgG per ml, the specific agglutination was most rapid and strongest (data not shown). Agglutination could be observed within 30 min. Sensitized blue latex beads in suspension were therefore prepared for the following experiments.

The results obtained by the two methods using brain and saliva specimens from the same dog for rabies virus antigen testing were as follows (each specimen was coded and tested blindly). Of 238 paired specimens tested, 80 were concordantly positive and 152 were concordantly negative. The remaining 6 paired samples showed discordant results (6 of 238 samples [2.5%]). Four were positive by FAT but negative by LA. The other 2 paired samples were negative by FA but positive by LA. The sensitivity, specificity, positive predictive value, and negative predictive value of the LA test were 95.2, 98.7, 97.6, and 97.4%, respectively.

Dogs are the principal transmitters of rabies virus in most of Asia. Transmission is almost exclusively via infected saliva. Virus appears in the saliva of dogs before and during the appearance of clinical signs (14, 17). There have been previous reports of apparently healthy dogs that intermittently excreted salivary rabies virus (5). In experimentally infected dogs, 70% developed the dumb (paralytic) form of rabies, 12% developed the furious (encephalitic) form, and 18% died without showing any signs of disease. Dogs showing clinical signs excreted virus in their saliva up to 14 days before such signs appeared (8). The dissemination of virus in tissues outside the central nervous system depended on the inoculum dose and the length of the incubation period. A large inoculum produced a short incubation period and a rapid course of illness leading to death. Alternatively, viral antigen was well distributed in many parts of the body of dogs that died after a long incubation period (6, 14). Virus was detected in salivary glands in only 25 to 40% of dogs inoculated with a large dose of virus, while almost all dogs inoculated with a small dose had virus in their salivary glands (7). In naturally infected dogs the rate at which virus was present in the salivary glands ranged from 75 to 100%. This suggests that the amount of virus introduced by a bite is small (8).

With the LA test, a false-positive result might be due to some components in saliva that affect the specific agglutination of sensitized latex. Saliva contains a small number of immune active and epithelial cells and small amounts of immunoglobulin, digestive enzymes, and bacteria of the normal flora (15). The components associated with false-positive reactions are not known. Nevertheless, the occurrence rate of false-positive reactions was rather low (2 of 238 samples [0.8%]).

Our findings showed that saliva samples could be used as an alternative to brain specimens for rabies virus antigen testing. Saliva collection is simple. In addition, it is safer, since occupational and disposal risks are eliminated when saliva is collected from dead animals. There may also be savings in cost and time. The collected saliva is stable at room temperature for a few days and remains so for longer periods when refrigerated or frozen. However, the load of infective virus in saliva is lower than that in brain. Furthermore, there is need for a method of confirming LA-negative results. Nevertheless, false-negative results occurred rarely in tested cases (4 of 238 samples [1.7%]).

We are grateful to Henry Wilde for his critical review of the manuscript and to Tamotsu Satoh for his generous gift of latex. We also thank Surasak Akesowan for preparing the horse immune serum and Veera Tepsumethanon for helpful discussion.

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