Special Articles

Laboratory Techniques for Rabies Diagnosis in Animals at QSMI

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The Queen Saovabha Memorial Institute (QSMI), Thai Red Cross Society was founded in 1922 to carry out the production of the nervous tissue vaccines, post-exposure prophylaxis (PEP) treatment, research, and laboratory diagnosis. The diagnostic laboratory had replaced the Seller's staining with direct fluorescent test (DFA) as standard technique in 1987 and the mouse inoculation technique (MIT) had been employed as the confirmatory test to ensure the result of the diagnosis. Other techniques conducted at our facility such as tissue culture infection and polymerase chain reaction techniques are less practical although the sensitivity is competitive with DFA but mainly for research references.

Keywords: *Rabies, Canine, Diagnosis, Veterinary, Fluorescent technique*

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The Queen Saovabha Memorial Institute (QSMI), Thai Red Cross Society was founded in 1922 in memory of Her Majesty the Queen Mother of HM King Vajiravudh to carry out the production of the early nervous tissue vaccines, post-exposure prophylaxis (PEP) treatment, research, and laboratory diagnosis.

The direct microscopic examination of brain tissue for Negri bodies was obtained as standard service. Seller's stain containing methylene blue and basic fuchsin on the impression brain smears had been used to demonstrate the cytoplasmic inclusion bodies. Regarding other pathogens such as canine distemper virus and canine hepatitis virus that also produced cytoplasmic inclusion bodies and the artifacts can be difficult to distinguish Negri body although those are smaller in size by Seller's stain. This led to a number of false positives. Specificity and accuracy in rabies diagnosis had been improved since Goldwasser and Kissling in 1958[1] introduced the fluorescent antibody technique. QSMI's diagnostic laboratory had replaced the Seller's staining with direct fluorescent antibody test (DFA) as standard technique in 1987 and the mouse inoculation technique (MIT)[2] had been employed as the confirmatory test to ensure the result of the diagnosis. QSMI was then designated as the WHO collaborating center for research on rabies pathogenesis and prevention since 1994 up to the present with continually periodic evaluation. QSMI is regarded as Thailand's most important diagnostic laboratory testing one-third of the country's specimens from the Central part of Thailand. Data shown in Fig. 1 displays the proportion of positive found specimens compared with the total number.

Dogs are the main vector of rabies in Thailand. Ninety-five percent of samples tested at our laboratory are of dog specimens, 3% are of cats, the rest are of wildlife and accidentally infected farm animals. The causative agent "Lyssavirus genotype-1" is a neurotropic virus commonly transmitted through the bite of an infected animal. The virus is usually introduced at the site of inoculation to the central nervous system (CNS) via peripheral nerves. The Ammon's horn or Hippocampus is the preferred area of the brain for the demonstration of Negri bodies while the brain stem is the area preferred for demonstration of viral antigen (by DFA)[3,4] or RNA in recently applied molecular diagnostic techniques. Viral antigen and RNA can also be found from salivary glands, skeletal muscle at the bite site, pancreas, cornea, heart muscle, mammary glands. RNA can be recovered by molecular techniques in body fluids such as saliva, cerebrospinal fluid (CSF), tear, urine.

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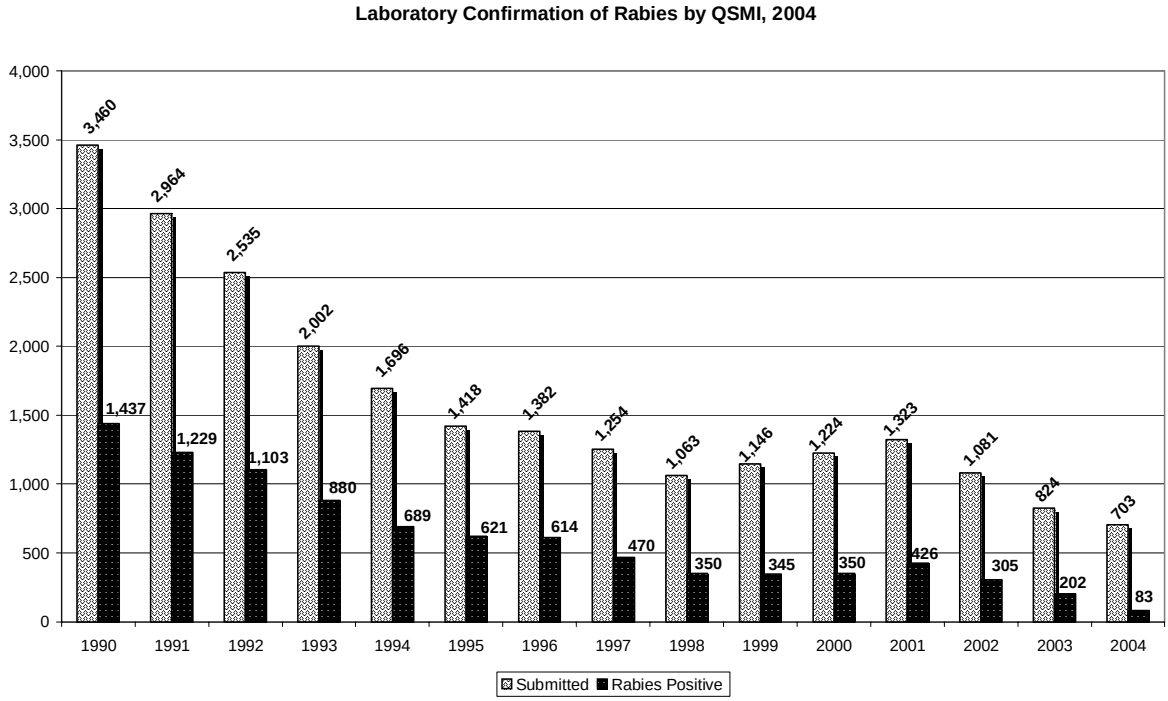

Fig. 1 Laboratory confirmation of rabies by DFA conducted at QSMI 1990-2004

Diagnosis of animal rabies can be carried out in 2 settings: on live or dead animals. Examination of suspicious live animals can be observed by veterinary specialists but possible specimens such as saliva, CSF, urine may be collected for laboratory confirmation and usually engage more time and less accuracy. Laboratory diagnosis from the carcass varies according to the type of specimens as characterized in Table 1. However, the brain is the most appropriate organ or tissue for rabies viral identification using DFA[3]. DFA provides high specificity and accuracy and used as the standard technique for rabies diagnosis worldwide. Other methods conducted at our laboratory such as mouse inoculation technique (MIT), tissue culture infection techniques, both of which are less practical although the sensitivity is competitive with DFA.

Detection of specific humoral antibodies is not usually helpful in animals with previous rabies vaccination history and early manifestations of the disease. The latter usually is not associated with an antibody response which only appears after the animal is kept alive by artificial means.

Eradication of the carcasses after specific tissues have been collected should be mentioned carefully. An electronic ignited fuel supplied incinerator has been used at our diagnosis facilities. The incinerator working temperature is between 360-400 celsius at the main chamber where carcasses are burnt

Table 1. Laboratory techniques for rabies diagnosis at QSMI facility

Technique	Type of specimen	Advantage/ Disadvantage
Direct Fluorescent Antibody Technique (DFA)	Target organs, such as brain, salivary glands, liver, spleen, pancreas, nuchal skin, although brain is the most appropriate sample	Applicable with most tissue sources Not applicable in decomposed tissue
Mouse Inoculation Technique (MIT)	Similar to DFA	Only use fresh tissue
Tissue Culture Infection Technique (TCIT)	Similar to DFA	Only use fresh tissue
Polymerase Chain Reaction (PCR)	Similar to DFA including body fluids; saliva, CSF, urine	Applicable in all tissue conditions Expensive Need experienced technicians

directly by the flame and the second chamber where the incomplete gas has been oxidized with the second burner.

Laboratory Techniques for Testing Rabies at QSMI

Direct Fluorescent Antibody (DFA) Technique

The immune reacting test is currently the best diagnostic tool for demonstration of the viral antigens. Combined accuracy and speed of this technique was introduced by Goldwasser and Kissling in 1958. The result can be obtained within 1-2 hours. Impression smears of fresh brain tissue from hippocampus, brain stem, and cerebellum are stained with fluorescein labeled antibodies. When the rabies-antirabies specific complex is formed, the fluorescein stain coated on the surface of antibodies will be examined by fluorescent microscope. According to the high specificity, accuracy and low cost, it is recommended worldwide as a standard technique in diagnosis of rabies[5] by the world health organization (WHO). All of the 33 laboratories in Thailand carry out this standard technique.

Demonstration of viral antigen with DFA from frozen nuchal skin and corneal impression smears are not practical in animals since DFA testing on brain tissue provides greatest accuracy. At present, the specimens preserved in formaldehyde can be examined by DFA when treated with proteolytic enzymes prior to the staining process but not widely serviced as standard protocol.

Mouse Inoculation Technique (MIT)

Another technique recommended by WHO uses mice between 3-6 weeks of age. Mice will be observed for 30 days for the presence of clinical signs after intra-cerebral injection of 20% tissue suspension. If mice die of rabies infection, rabies virus can be identified by DFA test for the detection of viral antigen in the brain. According to the length of observation period, this technique is employed for confirmation.

Tissue Culture Infection Technique (TCIT)

An in vitro laboratory test with susceptible tissues to rabies infection such as murine neuroblastoma cells (MNA) or baby hamster kidney cells (BHK-21) suspended in agarose[2,6]. Propagation of rabies virus in the tissue produces cytoplasmic inclusion bodies which are observed by DFA stain. Some laboratories have replaced the MIT with TCIT for rabies viral demonstration. This test may not be reliable if the source specimens are decomposed or contaminated.

Polymerase Chain Reaction (PCR)

Molecular techniques demonstrate genetic substrates of the target virus and are well adapted for testing in tissue or fluid with high accuracy and sensitivity. A very small amount of specimen is required. Fresh specimen is preferred to decomposed ones. PCR can yield positive result in decomposed specimens, however, the result is not very consistent or reliable. According to the expensive equipment and single use tools causing high expenses, it is limited to confirmation of human rabies.

Amplification of template DNA (after reverse transcription step) with polymerase enzyme from 1 pair of specific primers in thermocycle engine. This technique consists of 3 major processes; denaturation, annealing, extension. The amplified product will then be characterized with a gel-electrophoresis technique and is compared with a known control. The nested PCR technique[7] which is the double step amplification was introduced to increase sensitivity.

It should be noted that viral RNA secretion in saliva, urine and cerebrospinal fluid is intermittent. One negative test, whether DFA, TCIT, nested PCR or other recommended molecular techniques must not be considered reliable for diagnosis and must be repeated.

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การวินิจฉัยโรคพิษสุนัขบ้าในสัตว์ ณ ห้องปฏิบัติการสถานเสาวภา

บุญเลิศ ล้ำเลิศเดชา

สถานเสาวภาก่อตั้งในปี พ.ศ. 2465 โดยมีวัตถุประสงค์หลักเพื่อการผลิตวัคซีนที่เตรียมจากเนื้อเยื่อของระบบประสาท การให้บริการฉีดวัคซีนแก่ผู้สัมผัส งานวิจัยเกี่ยวกับโรคพิษสุนัขบ้า และ การวินิจฉัยโรคพิษสุนัขบ้าทางห้องปฏิบัติการ ในปี พ.ศ. 2530 การย้อมสีตามเทคนิคของ Seller เพื่อการวินิจฉัยได้ถูกยกเลิกโดยสิ้นเชิง และใช้เทคนิคฟลูออเรสเซนต์แทน ซึ่งเป็นวิธีมาตรฐานที่ทั่วโลกยอมรับ นอกจากนี้ยังใช้เทคนิคการเพาะเชื้อในสัตว์ทดลองเพื่อยืนยันผลการทดสอบ สำหรับเทคนิคอื่น ๆ เช่นการเพาะเลี้ยงเนื้อเยื่อ และการเพิ่มจำนวนสารพันธุกรรมไม่ได้นำมาใช้ในงานบริการทั่วไป แต่ใช้เป็นวิธีทดสอบยืนยันสำหรับงานศึกษาวิจัย