Chapter V

Discussion

At present, diagnossis of animal rabies by direct immunofluorescence is rapid, highly sensitive and specific but it requires a costly and well-maintained fluorescent microscope and experienced personnels. The confirmatory test, MIT, although also highly sensitive and specific, but it is time-consuming and requires an animal house facility.

The purpose of this study is to develop a new technique, dot-immunoblot. The method is simple, rapid and cheap, and thus hoped to replace the time consuming MIT as well as to enable rabies dignosis antemortemly for detection of rabies virus antigen in the salivary gland suspension and saliva. It can be accomplished within 5-6 hours and without sophisticated equipments nor highly trained technicians are required.

The technique of dot-immunoblot developed in this study followed the method of Lakeman et al for the detection of herpes simplex virus antigen in cerebrospinal fluid (11). Several blocking agents were tried including various concentrations of bovine serum albumin, 0.1% PBS-T and BLOTTO. We found that 5% BLOTTO (W/V), in agreement with Lakeman et al, was the best blocking agent for dot immunoblot.

Although supplementary serum was not used in the test developed by Lakeman et al, we found that a mixture of 2% goat

serum (GS), 5% chick serum (CS) and 0.002% normal human serum (NHS) was needed in the diluent of the primary and secondary antibodies in order to block the non-specific reaction. One of the reasons for this difference may be due to the fact that the antibodies used in our study were polyclonal antibodies, thus may result in more non-specific background as compared to the monoclonal antibody used by Lakeman et al (11).

Different primary and secondary antibodies required different combinations of supplementary serum. For example, all 3 sera were needed for the primary antibody, RRIG but only GS and NHS were required for the primary antibody, ERIG-BBL and only GS and CS for both types of secondary antibody. The reason for this difference is unknown. It may be due to the difference in the purity of each reagent used. As a matter of fact, GS has been routinely added in the reagents of the commercial ELISA kits (Organon).

At first, the self-prepared RRIG was used, but in order to make the test more widely applicable, the commercially available primary antibody, ERIG-BBL was also studied. It was found that ERIG-BBL as compared to RRIG gave a higher color intensity if the test was positive and a clearer background if the test was negative. This may be due to its higher antibody level as well as purity. Therefore, although ERIG-BBL was slightly less sensitive and less specific than RRIG on stored parotid gland suspensions and saliva, (i.e.,81.8 % vs 72.7% for sensitivity and both 85.7 % for specificity), but for the sake of convenience, it was suffice to be recommended for general use, especially for unstored specimens.

The results of our study using RRIG showed that all freshly prepared parotid gland suspensions gave 100% sensitivity and specificity on dot immnoblot as compared to FAT and MIT. With fresh saliva, the sensitivity and specificity of dot immunoblot were 94.7% and 91.6% respectively. The slightly less sensitivity and specificity of the saliva immunoblot as compared to parotid gland suspension immunoblot may be caused by several factors. Firstly, there might be no viral excretion or very small amount of virus was excreted into the saliva even the virus was abundantly present in the parotid glands. Secondly the saliva might contain dirt, chromogens or enzymes as released from the hemolysis of blood-contaminated saliva (e.g. endogenous peroxidase) which might interfere with the color development.

The study in stored specimens showed that the sensitivity was reduced to 96.5% for parotid gland suspension immunoblot and 89.6% for saliva immunoblot. The specificity was also reduced to 84% and 73% for parotid gland suspension and saliva immunoblots respectively. The less sensitivity and specificity of the stored specimens might result from the denaturation and decomposition of the antigen.

In addition to the data reported in this study, we encountered additional 4 dogs with positive FAT but negative MIT, using brain specimens. All were positive on parotid gland suspension and saliva immunoblots and the results were confirmed by positive FAT on parotid glands and positive MIT on parotid gland suspension as well as on saliva. This may indicate that brain FAT is more sensitive than MIT and salivary gland suspension and saliva

immunolblots are as sensitive as brain FAT. However, this may not be ture because the unproperly preserved brain may decompose and the dead virus will result in negative MIT whereas the FAT detection is still positive because no viable virus is required for FAT.

Furthermore, although FAT is already highly sensitive, but false negative results can be observed. Data from the Diagnostic Unit of QSMI indicated that 11 out of 2191 FAT-negative brains were MIT positive. In this study, we observed one FAT-negative, MIT-positive dog which was immunoblot positive on parotid gland suspension.

This observation agrees with other reports that FAT is less sensitive than MIT .i.e., 98% as compared to MIT. This may be due to the small number of viral particles in the rabid brain, of which multiplication in the brain is needed for its detection by MIT.

Taken together, before the superiority of salivary gland suspension and saliva immunoblots over brain FAT and MIT can be firmly established, a study of a much larger specimens have to be carried out.

The study of Vaught et al (40) showed that 13% of the dogs which survived the experimental of rabies infection intermittently excreted rabies virus in saliva although virus was present in the salivary glands at all time

The presence or absence of rabies virus in the saliva may partly depend on the time of sampling. The absence of virus in the parotid gland does not necessarily mean that the saliva is non-

infectious bacause the virus may be excreted from the other salivary gland such as maxillary or submandibular glands. Therefore,negative test with parotid gland and saliva do not imply, without exception,that virus is not present in the brain.

With the above reasoning, brain still remains to be the ultimate tissue to be used in rabies diagnosis. We, therfore, extended our dot immunoblot study to the brain tissues. Similar to the results with stored salivary gland suspension and saliva, stored brain suspension was also less sensitive (75 - 61.9%) and specific (100 - 95.2 %). However, when freshly prepared brainstem suspensions were used in immunoblot, the sensitivity and specificity could be increased to 100% and 88.8% respectively with ERIG-BBL and 94.7% and 88.8% respectively with RRIG. The reason for this improved sensitivity and specificity may be due either to fresh nondegraded state or to the viral predilection in the brainstem . The latter assumption needs to be firmly established becaused it posesses important practical implications. If brainstem is indeed better than the whole brain in FAT, MIT and dot immunoblot, it should be the only region of the brain submitted for rabies diagnosis.

Antemortem demonstration of rabies virus in the quarantined animals, as early as possible, will markedly improve the success of rabies prophylaxis while minimizing the cost of unnecessary immunization. This formed the basis of our prospective study of rabies virus excretion in the saliva of quarantined dogs. Although the technique of collecting saliva was difficult and cumblesome, the results were very satisfactory. All of the 6 dogs

subsequently proved rabid excreted the virus in the saliva which could be detected 1-3 days before deaths. Sequential follow up of these dogs were impossible because of the early death. In addition, of all saliva specimens sequentially collected from 4 quarantined dogs which finally proved nonrabid, none was positive on dot-immunoblot , giving a 100% sensitivity and specificity of the test. However, the method of saliva collection may play an important role on the test result. We have observed 3 positive specimens out of 10 saliva specimens from 10 healthy pet dogs which were collected by 10 different individual using non-standardized methods , such as by direct suction from the mouth (data not shown).

Such a possibly, false-positive results may be due to the nonspecific staining of the protein in the concentrated saliva because the test became negative when saliva was diluted 100 folds. Similar dilution of the truely positive saliva specimens still yielded positive results.

In summary, our results indicated that dot-immunoblot technique , when properly performed could be of great diagnostic value in rabies diagnosis both antemortemly and postmortemly. For antemortem diagnosis using the saliva from the biting animals, the results will aid in making the decision for early post-exposure rabies prophylaxis. For postmortem diagnosis, we feel that saliva, salivary glands or brainstem or any combinations of the above 3 specimens can be reliably used with the dot-immunoblot technique. After a more systematic, double-blind, large-scale comparative study, it may be able to replace the time-consuming MIT and the FAT in the laboratories where fluorescent microscope is not available or rabies diagnostic facility has not yet been set up.

Conclusions

1. The technique of dot - immunoblot for the detection of rabies virus antigen has been developed.

2. The method could be used to detect rabies virus antigen in parotid gland suspension and saliva with high sensitivity and specificity in correlated with the conventional FAT and MIT of the brain.

3. The status of the specimens,stored or freshly used, may influence the test results. The freshly prepared specimens were required.

4. The use of commercial antirabies antibody (ERIG-BBL) as the primary antibody in our dot-immunoblot technique gave a high contrast intensity between negative and positive specimen. The need or the important of the other antirabies antibodies in the detection of various antigenic components of the virus in different tissues remained to be investigated.

5. The study of viral excretion in saliva of quarantined dogs with this method gave an excellent correlation with the other standard diagnostic methods and with clinical picture of the animal in both rabid and nonrabid quarantined animals

6. The current diagnostic tests of rabies include SS,FAT, and MIT using brain specimens. SS is the least sensitive among all and therefore,should be abandoned although it is still preferred by many investigators as additional supportive evidence for FAT which is performed simultaneously. FAT and MIT are methods of high sensitivity and specificity but this cordant results can occur. In the case of negative FAT , one has to wait for a long time before the MIT result is known, thus, the post-exposure treatment may be

unnecessarily delayed. Therefore, saliva ,salivary gland or brain dot- immunoblot should be used in place of brain MIT.

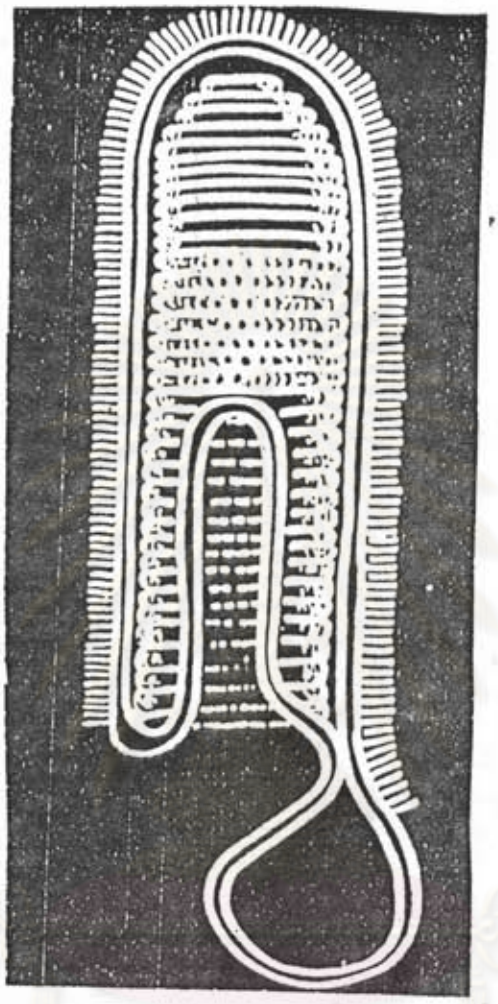

Figure 1 Diagrammatic representation of rabies virus
(Natural History of Rabies. Vol.1, G.M.Baer editor)

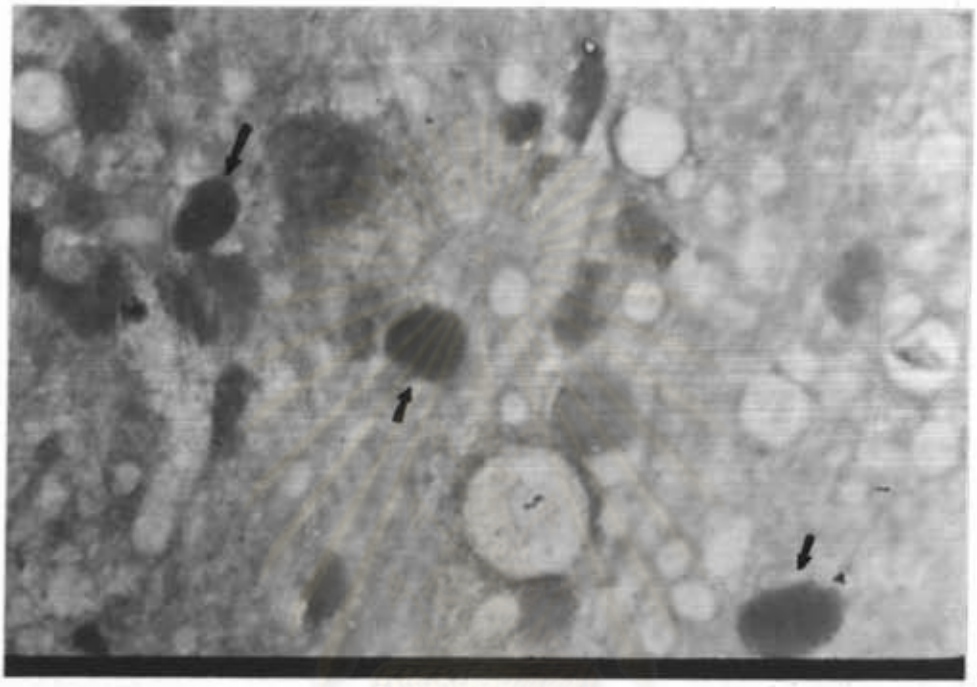

Figure 2 Negri bodies demonstration by Seller's stain. The Negri bodies take up the fuchsin and stained magenta or cherry red with variable in size and morphology. The rabies specific inclusion bodies , basophilic dark-blue staining granules must be present (x 100)

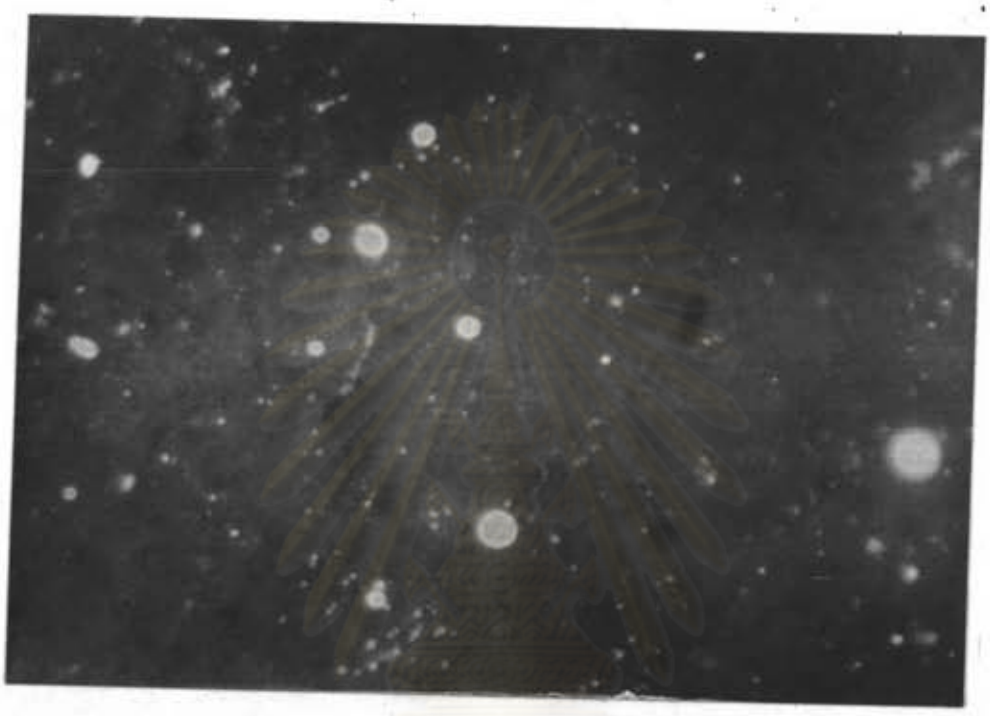

Figure 3 Fluorescent Antibody Test (FAT) or brain impression smear. Fluorescent material may vary from dustlike particles of < 1 um to large masses or threads of 2 to 10 um in diameter. (x 400)

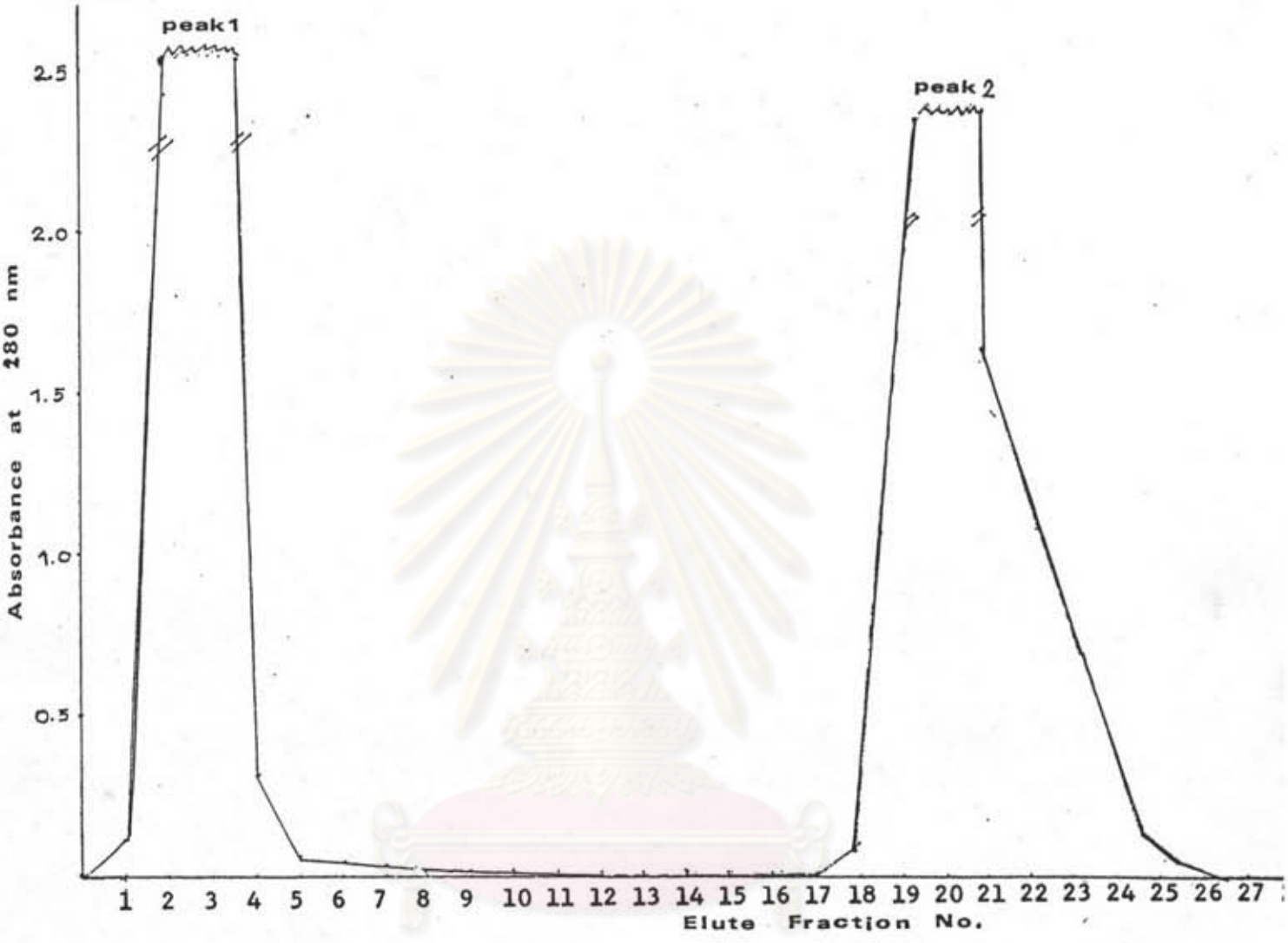

Figure 4 Affinity chromatography of rabbit antirabies on protein-A sepharose CL-4B column. The column was e luted with 0.05 M acetate in 0.15 M NaCl, pH 4.3 flow rate 50 ml/hr ; volume of fraction 3 ml

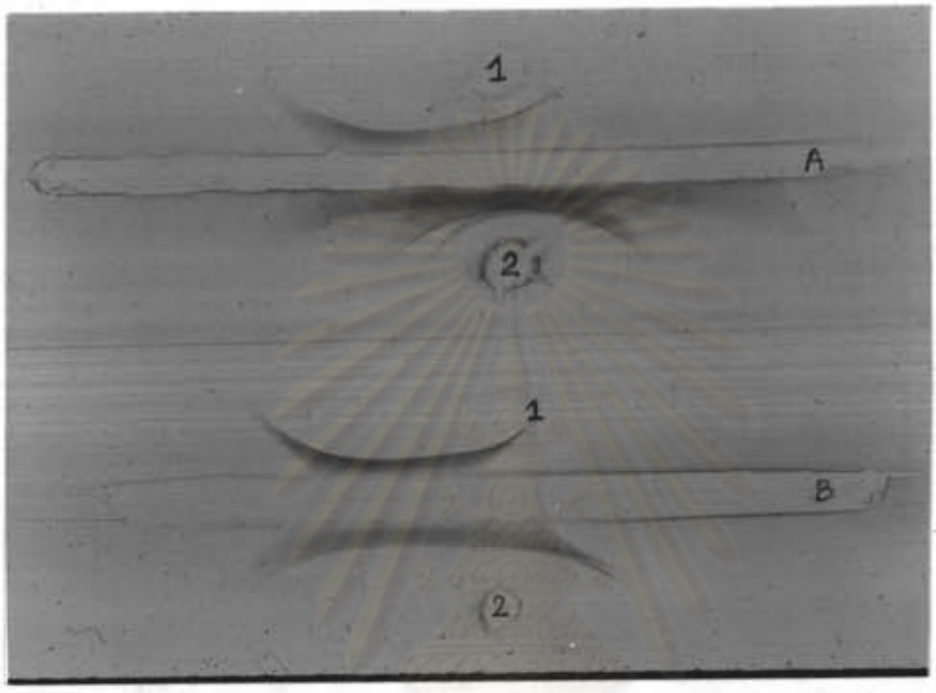

Figure 5 IEP of rabbit antirabies IgG (well 1) against

A swine antirabbit serum (trough)

B Goat antirabbit IgG (trough)

Central Normal rabbit serum (well 2)

Figure 6 Dot-immunoblot for detection of rabies virus antigen

Positive : blue dot

Negative : absence color

Table 1 Checkerboard titration to determine the optimal dilution of antibodies.
A. Between primary Ab rabbit antibodies IgG and secondary Ab biotin- conjugated goat anti-rabbit IgG (1 mg/ml)

Antigen	Secondary Ab dilution	Primary Ab (dilution) 1:50	1:100	1:150	1:200
PVRV 10^{-4} (0.1%PBS-T)	1:100	+4 (MOD)	+4 (MOD)	+2 (SL)	+1 (SL)
	1:200	+4 (MOD)	+3 (SL)	+1 (SL)	- (NB)
	1:300	+3 (MOD)	+3 (NB)	+1 (NB)	- (NB)
	1:400	+2 (MOD)	+1 (NB)	- (NB)	- (NB)

(a) Intensity of stain : - negligible, +1,+2,+3,+4 maximum obtained

(b) background MOD = Moderate.
SL = Slighly.
NB = No background

Table 1.B. Between primary Ab - equine antirabies globulin- BBL and Secondary Ab - biotin conjugated rabbit anti-horse IgG (0.7 mg/ml)

Antigen	secondary Ab	Primary Ab dilution 1:100	1:200	1:300	1:400
PVRV 10^{-4} (0.1% PBS-T)	1:100	+4 (MOD)	+3 (MOD)	+3 (MOD)	+3 (MOD)
	1:200	+4 (MOD)	+3 (MOD)	+3 (SL)	+2 (SL)
	1:300	+4 (SL)	+3 (NB)	+2 (NB)	+1 (NB)
	1:400	+3 (SL)	+2 (NB)	+1 (NB)	- (NB)

(a) Intensity : - negligible, + 1 , 2 , + 3 , + 4 maximum obtained

(b) Background : MOD = moderate

SL = slightly

NB = no background

Table 2 A The specificity and sensitivity of rabbit anti-rabies IgG (RRIG, 1:100 dilution) and biotin-conjugated goat anti rabbit IgG (1:300 dilution)

Antigen	Dilution of antigen						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
PVRV (40 mg/ml)	+ 4	+ 4	+ 3	+ 3	+ 2	+ 1	-
PCEC (8 mg/ml)	+ 2	+ 1	-	-	-	-	-
Rhabdomun (2.4 mg/ml)	+ 2	+ 1	-	-	-	-	-
HBs Ag (2.8 mg/ml)	-	-	-	-	-	-	-
HSV-2 (3.8 mg/ml)	-	-	-	-	-	-	-
Hela cell (3 mg/ml)	-	-	-	-	-	-	-
Vero cell (2 mg/ml)	-	-	-	-	-	-	-

Intensity : - negligible, + 1, + 2, + 3, + 4 maximum obtained

Table 2 B The specificity and sensitivity of equine anti-rabies globulin-BBL, (1:200 dilution) and biotin conjugated rabbit anti-hose IgG (1:300 dilution)

Antigen	Dilution of antigen						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
PVRV (40 mg/ml)	+ 2	+ 1	-	-	-	-	-
PCEC (8 mg/ml)	+ 2	+ 1	-	-	-	-	-
Rhabdomun (2.4 mg/ml)	+ 3	+ 3	+ 2	+ 1	-	-	-
HBs Ag (2.8 mg/ml)	-	-	-	-	-	-	-
HSV-2 (3.8 mg/ml)	-	-	-	-	-	-	-
Hela cell (3 mg/ml)	-	-	-	-	-	-	-
Vero cell (2 mg/ml)	-	-	-	-	-	-	-

Intensity : - negligible, + 1, + 2, + 3, + 4 maximum obtained

Table 3 Determination of working dilution of avidin Biotin-conjugated HRP (ABC)

	Antigen	ABC dilution 1:500	1:1000	1:2000	1:300
PVRV	10^{-4}	+ 4	+ 4	+ 3	+ 2
	10^{-5}	+ 4	+ 3	+ 2	+ 2
	10^{-6}	+ 4	+ 2	+ 1	+ 1
HBsAg	10^{-1}	+ 2	-	-	-
	10^{-2}	+ 1	-	-	-
	10^{-3}	+ 1	-	-	-
0.1% PBS-T		-	-	-	-

Intensity : - negligible, + 1, + 2, + 3, + 4 maximum obtained

Table 4 The results of dot-immunoblot on freshly prepared salivary gland suspensians and saliva from suspected dogs.

	Positive dot-immunoblot (a)	
MIT & FAT (BRAIN)	salivary gland suspensions	saliva
POSITIVE	19/19 (b)	18/19
NEGATIVE	0/15	1/12
Sensitivity	100%	94.7%
Specificity	100%	91.6%

(a) Rabbit antirabies was used as primary antibody

(b) Number positive per number examined

Table 5 The results of dot-immunoblot examination of stored salivary glands, saliva and brains from suspected dog by using RRIG

MIT & FAT (BRAIN)	salivary gland	saliva	brain
POSITIVE	28/29[a]	26/29	13/21
NEGATIVE	4/25	6/25	1/21
Sensitivity	96.5%	89.6%	61.9%
Specificity	84%	73%	95.2%

(a) Number ppositive per number examined

Table 6 The results of dot-immunoblot examination of stored salivary glands, saliva and brains from suspected dog by using ERIG - BBL

MIT & FAT (BRAIN)	salivary gland	saliva	brain
POSITIVE	18/22[a]	16/22	18/24
NEGATIVE	3/21	3/21	0/23
Sensitivity	81.8%	72/7%	75%
Specificity	85.7%	85.7%	100%

a) Number positive per number examined

Table 7 Result of dot-immunoblot of freshly brainstem by using RRIG and ERIG

FAT & MIT (BRAIN)	NO.	Positive dot-immonoblot on brain stem	
		ERIG	RRIG
POSITIVE	19	19	18
NEGATIVE	9	1	1
Sensitivity		100%	94.7%
Specificity		88.8%	88.8%

Table 8 Results of the study of viral excretion in the saliva of quarantined dogs.

		Brain		Saliva	
		FA	MIT	MIT	Dot-Immunoblot
rabid dog	1	+	+	+	+
	2	+	+	+	+
	3	+	+	+	+
	4	+	+	+	+
	5	+	+	+	+
	6 - 1[(a)]	+	+	+	+
	- 2	+	+	+	+
Non-rabid dog	1 - 1	ND	ND	-	-
	- 2	ND	ND	-	-
	2 - 1	ND	ND	-	-
	- 2	ND	ND	-	-
	- 3	ND	ND	-	-
	- 4	ND	ND	-	-
	3 - 1	ND	ND	-	-
	- 2	ND	ND	-	-
	4 - 1	ND	ND	-	-
	- 2	ND	ND	-	-
	- 3	ND	ND	-	-
	- 4	ND	ND	-	-

\+ Presence of blue dot
\- Absence of blue dot
ND = Not done
(a)= serial specimens examined