CHAPTER IV

RESULTS

Isolation of Cytoplasmic RNA

Efficient extraction of RNA from the postnuclear cytoplasmic lysate is conducted by denaturation of proteins with SDS and urea. The proteins were separated by organic solvent extraction, and RNA was collected in ethanol precipitate form. Isolation of cytoplasmic RNA from rabies-infected and uninfected BHK cells was performed as described in materials and methods, whereas the RNA from infected and uninfected canine brain was prepared as mentioned in RNA preparation for RNA-DNA dot hybridization. RNA was stored in 70% ethanol at -85 °C until use. Absorption of RNA preparation was measured at 260 and 280 nm. The ratio of $A_{260/280}$ was in the range of 1.75-1.90, indicating that RNA isolated by urea/SDS extraction method contained less contaminating proteins. The estimated yield was 1-1.3 μg/10^6 BHK cells or 1.5-2 μg /mg canine brain. Quality of the RNA preparations were proven on 0.7% agarose denaturing gel electrophiresis as shown in Figure 8. There are no signs of high molecular weight RNA degradation as judged by the uniform migration of ethidium bromide-stained RNA and the presence of 28S and 18S ribosomal RNA. The quality of RNA preparation was further proven by Northern blot hybridization. The RNA was transfered to Hybond-N^+ and hybridized with rabies oligonucletide probe specific for N gene. The 1.4-kb transcript of N-gene from rabies-infected cells and infected canine brain, migrated as distinct bands without sign of degradation, as illustrated in Figure 9 (lane 3 and 4, respectively).

PCR Amplification of Specific Regions of Rabies Genes

Portion of genomic cDNA of each individual rabies genes was constructed using the gene-specific primer pairs (in Table 2) and then PCR amplified as described in materials and methods. The results presented in Figure 10 showing the amplification of desired gene portions. The cDNA fragments of N, G, NS, L and M gene were approximately 462, 963, 628, 433 and 598 bp long, respectively (Figure 10, A) . None of the PCR products were obtained with RNA extracted from uninfected BHK-21 by any sets of primer pair (Figure 10, B). The amplified gene portions were confirmed by Southern blot analysis. The blot was cut into 5 pieces. Each

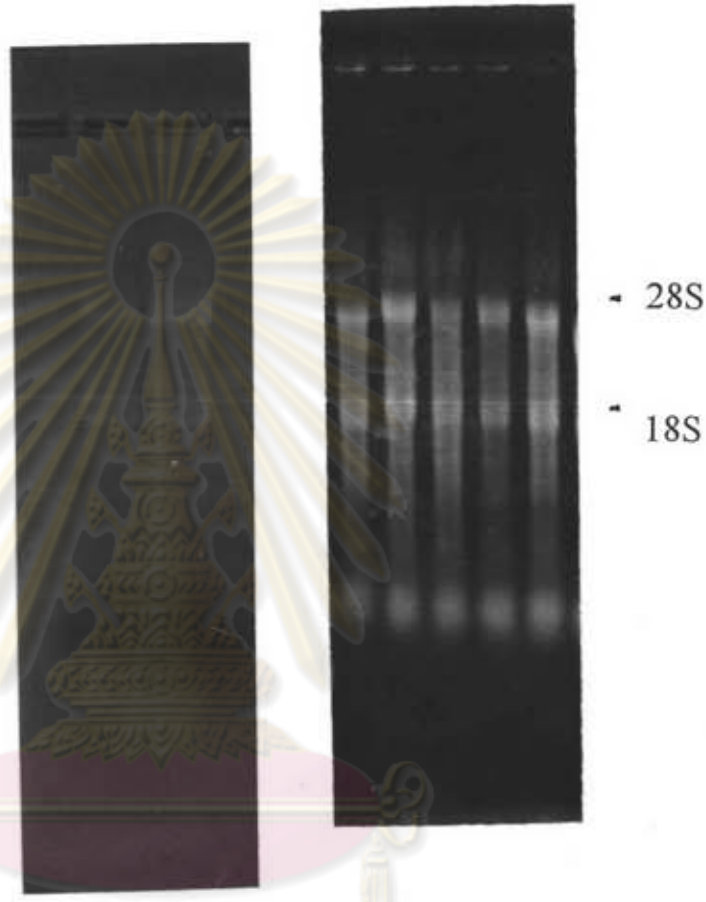


Figure 8. Denaturing agarose gel electrophoresis of RNA. Five of μg was electrophoresed through formaldehyde denaturing agarose gel and stained with ethidium bromide. Multiple prepartions of RNA isolated from rabies-infected BHK cells (A) are compared those RNA prepared from canine brian (B).

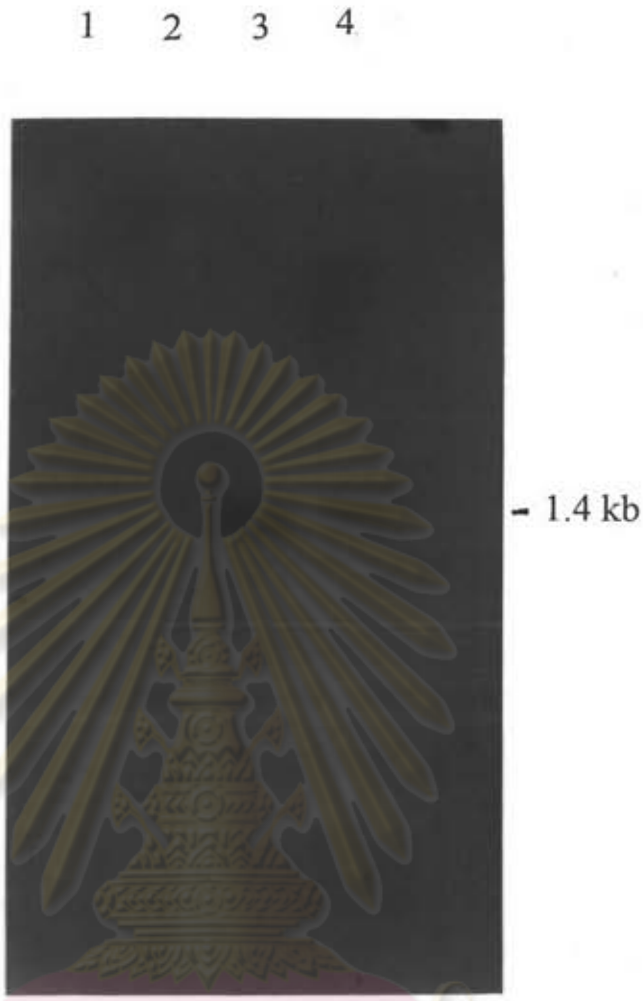


Figure 9. Northern blot analysis of RNA preparation. Five μg of cytoplasmic RNA were electrophoresed and transfered to nylon membrane. Rabies N-transcript was identified by Northern hybridization.

Lane 1, RNA from BHK cells; lane 2, RNA from non-infected canine brain; lane 3, RNA from rabies-infected BHK cells; lane 4, RNA from rabies-infected canine brain.

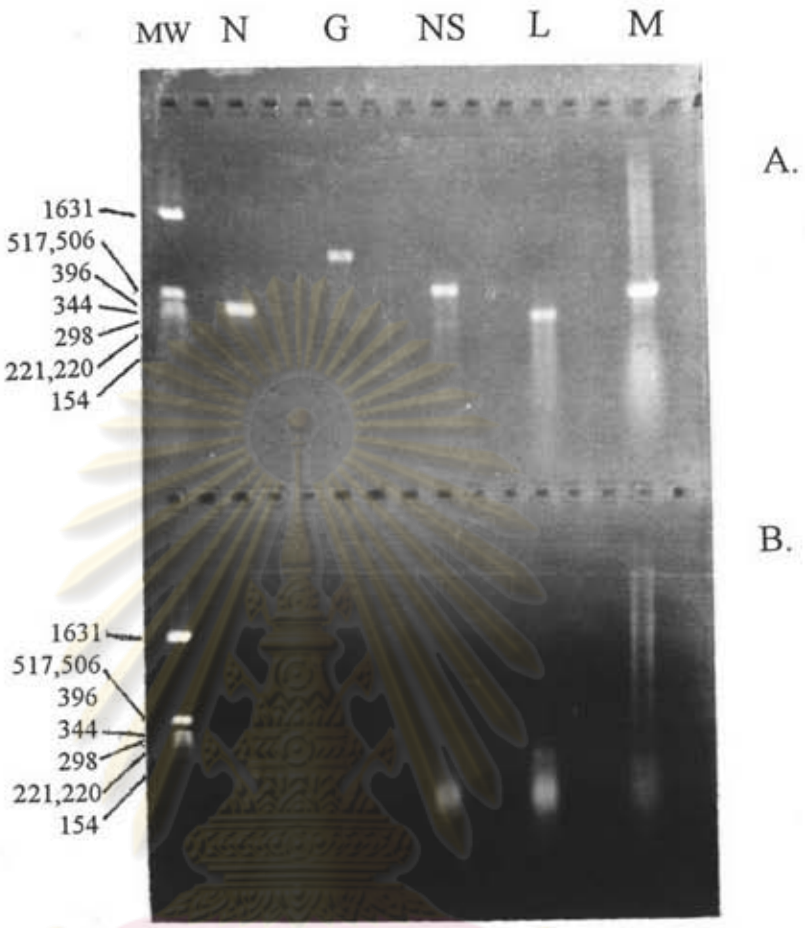


Figure 10. PCR amplification of rabies viral RNA. Portions of five viral genes were amplified using the gene specific primer pairs. The amplified products were analysed by 1.2% agarose-gel electrophoresis. Amplifications were performed with RNA of infected BHK cells (gel A) and RNA of non-infected cells (gel B). Reaction of lane N, G, NS, L, and M were performed by using the primer pairs for gene N, G, NS, L, and M, respectively. Lane MW is pBR322/*Hin*fI standard size marker.

piece contained the PCR-amplified product of N, NS, M, G or L gene. Then each blot was individually hybridized with its internal oligonucleotide probe as shown in Table 3. The results showed that the PCR-amplified products were the portions of N-, G-, NS-, L- and M-gene of rabies virus (Figure 11, A). No signals were detected from those PCR amplifications of uninfected BHK cells (Figure 11, B). In addition, specificity of the PCR amplification was also tested by using DNA and RNA samples from various sources. There were no amplifications with these 5 primer pairs when pGEM-3Z, *E. coli.* DNA, BHK-21 RNA, herpes simplex type I and type II, and papilloma virus RNA were used as templates (data not shown). As expected, each designed primer pair could be used for amplification of the cDNA fragment corresponding to each rabies gene sequence.

Construction of Rabies cDNA Probes

Cloning of specific DNA fragments complementary to 5 individual genes of rabies virus was acheived by RT-PCR technique as mentioned above. Since the primers were designed such that the amplified fragment contained an *Eco*RI site at its 5' end and a *Pst*I site at its 3' end, the amplified fragments could be digested with *Eco*RI and *Pst*I and sticky-end cloned into *Eco*RI/*Pst*I sites of pGEM3-Z plasmid vector. For an unexplained reasons, the amplified products were not able to be digested with neither *Eco*RI nor *Pst*I. The ability of restriction digestion was proven as follows. The 5' end of PCR products was [γ-^{32}P]-labelled by T_4 polynucleotide kinase and unincorporated [γ-^{32}P]-ATP was removed. The labelled PCR fragment was incubated with restriction endonucleases and the digestive products were analyzed by polyacrylamide gel electrophoresis and autoradiography. The elctrophoretic mobility showed a distinct band of labelled PCR-amplified fragment suggesting no sign of restriction digestion. To overcome this technical difficulty, the fragments were blunt-end cloned into *Sma*I site of pGEM-3Z. The PCR fragments were kinased and subjected to fill-in reaction to blunt end the DNA fragments. The pretreated PCR-amplified cDNA was ligated with *Sma*I-digested plasmids and were subsequently transformed into *E. coli* JM109 host cells and the cells were plated onto SOC agar containing IPTG and X-gal. White clonies were screened for the presence of cDNA fragments by PCR technique.

Ligation of blunt-ended PCR fragments to pGEM-3Z and transformation of JM109 resulted in 13-20 white, ampicillin-resistant colonies per ng of cDNA used in ligation reaction. The number of white colonies was estimated to be 90% of the total colonies. More than 70% of these transformants possessed rabies cDNA inserts as shown by PCR

Figure 11. Southern blot analysis of PCR amplification products. The PCR products in Figure 10 were transfered to nylon membrane and the blot was cut into pieces. Each containing individual PCR amplified gene product of rabies-infected (A) and uninfected BHK-21 RNA (B), respectively. Each blot was separately hybridized to the corresponding internal oligonucleotide probes resulting in fluorograms of corresponding gel A and B. PCR amplifications of N, G, NS, L and M gene were shown in lane N, G, NS, L and M, respectively.

screening (Figure 12). The size of PCR fragments obtained from preliminary screening of transformants was corresponded to the expected fragments of N, G, NS, L and M gene as described above. To test whether the inserts were derived from rabies genes, recombinant plasmids from 10 individual transformants were isolated and spotted on nylon membrane. Hybridization was carried out using internal oligonucleotide probe. Figure 13 confirmed that the inserts were indeed derived from rabies genes. Five cDNA clones : pRAB-N, pRAB-G, pRAB-NS, pRAB-L and pRAB-M were obtained; these were derived from rabies virus sequences belonging to the portions of N-, G-, NS-, L- and M-gene, respectively. The recombinant plasmids from these clones were used as probes for further study due to strong hybridization signal with rabies virus RNA and not with RNA from other sources.

Characterization of Rabies Recombinant Plasmids

The rabies clones (pRAB-N, pRAB-G, pRAB-NS, pRAB-L and pRAB-M) were cleaved with *Eco*RI and *Pst*I and analyzed through agarose gel electrophoresis as shown in Figure 14. The linearized pGEM-3Z plasmid vector was estimated to be 2.7 kb on 0.7% agarose gel. The inserts were detected to be 490, 1000, 660, 470 and 470 bp from recombinant plasmids containing the cDNA inserts of N, G, NS, L and M gene, respectively. The size of M-cDNA insert was reduced from 598 to 470 bp due to unexpected restriction site on PCR-amplified fragment of M-gene (restriction site at 5' and 3' end of PCR-amplified M-cDNA was not proven as described above). The restriction site was determined to be *Eco*RI. The DNA fragments were transferred to membrane and analyzed by Southern hybridization using each individual oligonucleotide probes. Results in Figure 15 proved that rabies recombinant plasmids were derived from 5 separated structural genes of rabies genome. Alternatively, no hybridization signal was detected when PCR-amplified fragments were hybridized to other gene-specific oligonucleotide probes (data not shown). This result suggested that no cross-hybridization was detected between these cDNA recombinant plasmids.

Specificity and Sensitivity of Rabies cDNA Probes

Dot hybridization was employed in the determination of sensitivity (detection limit) and specificity of the probes. The entire recombinant probes were labelled using ECL-random prime system and hybridized with various amounts of RNA from rabies-infected BHK cells. As shown in Figure 16-17 (picture A), the pRAB-NS and pRAB-L probes were able to

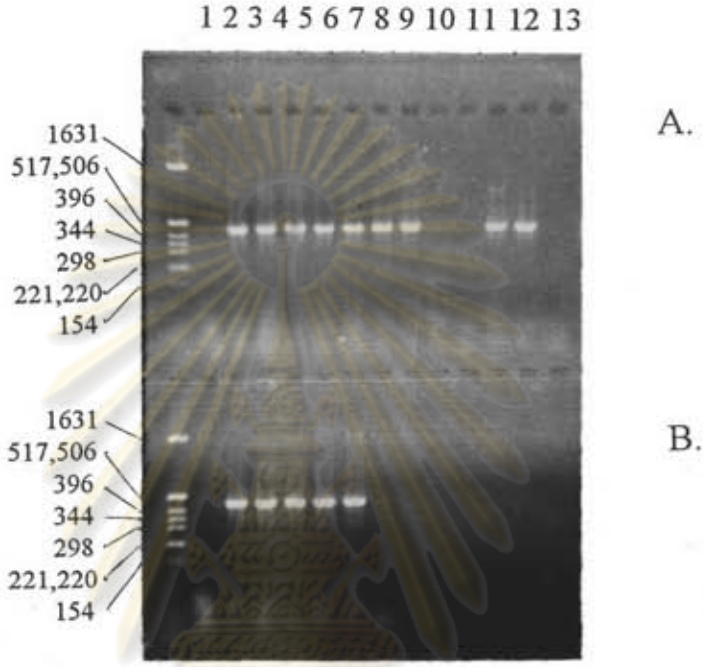


Figure 12. PCR screening for transformants carrying rabies M-cDNA sequence. Nineteen transformants were lysed and analyzed the gene portion by PCR. Amplified products were identified on 1.2% agarose gel electrophoresis (lane A1-A13 and B1-B7). Standard size marker is pBR322/*Hin*fI.

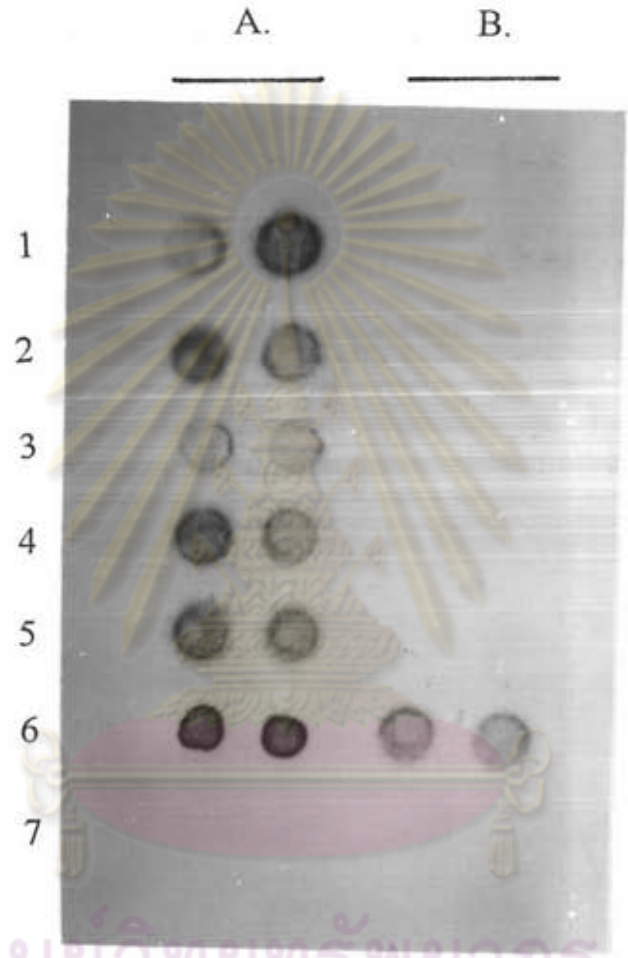


Figure 13. Dot-hybridization analysis of transformants. Plasmid from 10 individual clones of M-gene were isolated, spotted onto membrane and hybridized to oligonucleotide M-probe. A1-A5 and B1-B5 are plasmids isolated from individual colonies. A6 is PCR amplified positive control and B6 is RNA from infected BHK-21. A7 is PCR amplified negative control whereas B7 is RNA from unifected BHK-21.

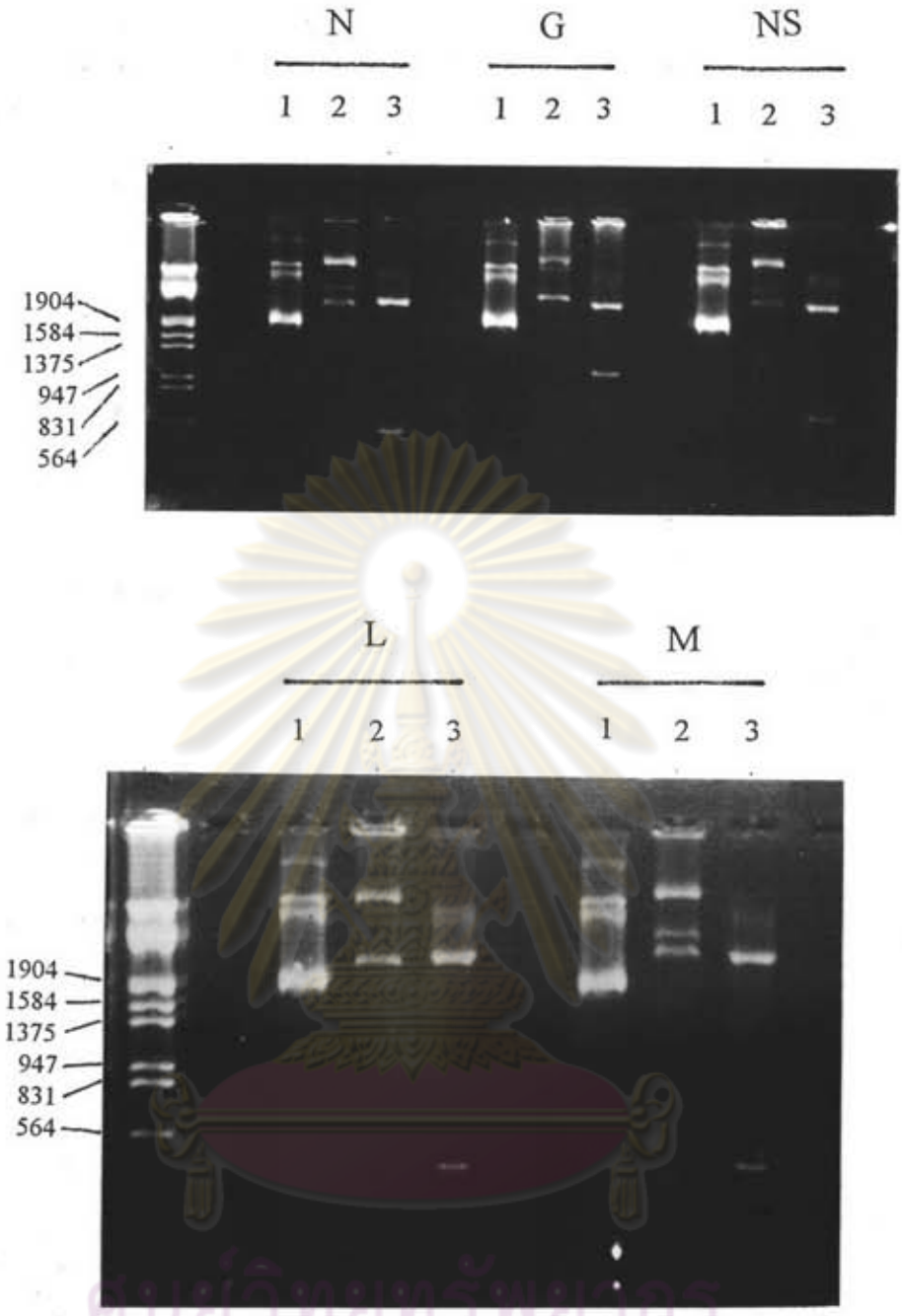
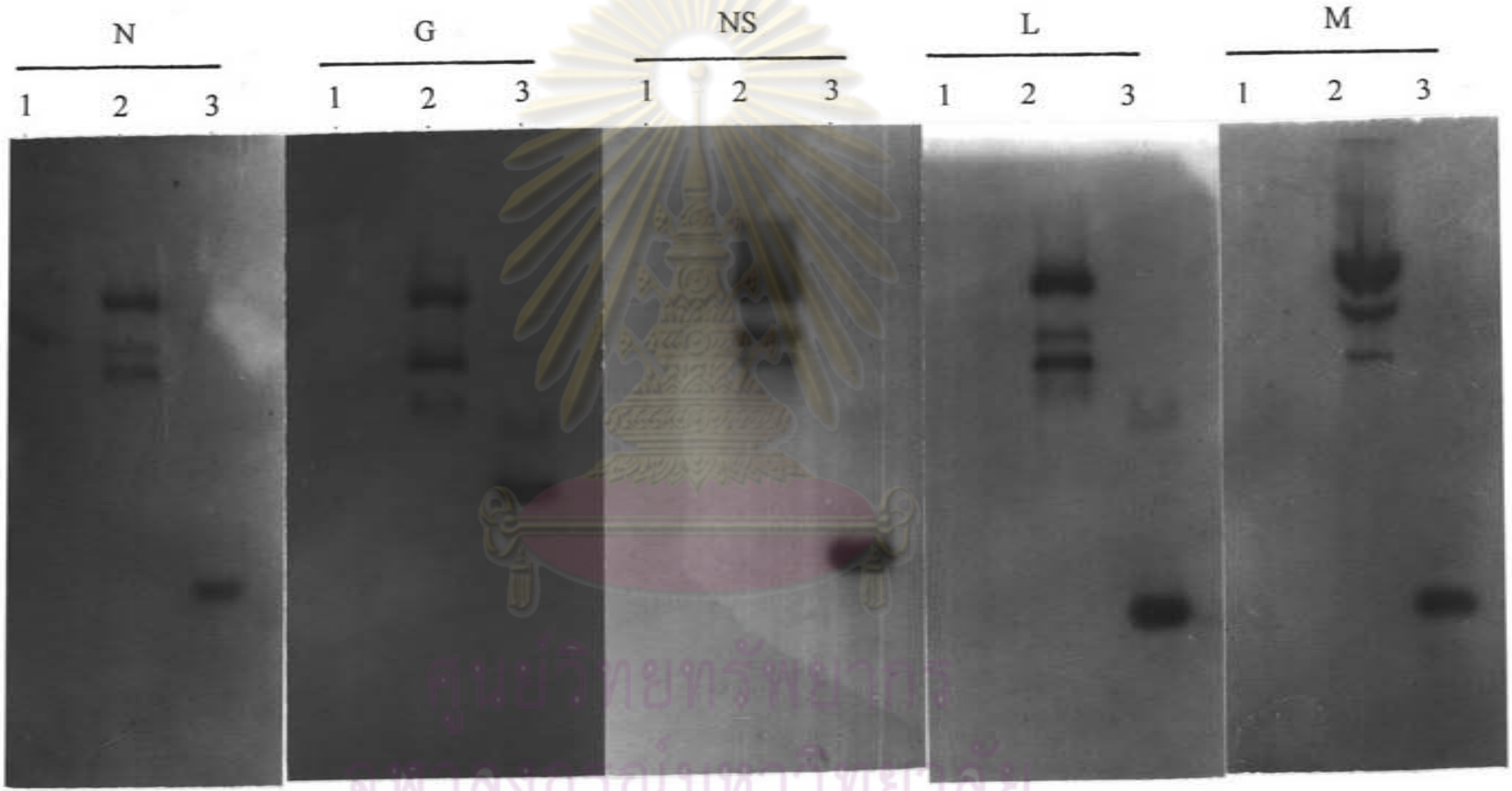


Figure 14. Agarose-gel electrophoresis of rabies recombinant plasmids digested with *Pst*I and *Eco*RI. Lane 1, 2 and 3 contain undigested pGEM-3Z, undigested recombinant plasmids and *Pst*I/*Eco*RI-digested recombinant plasmid, respectively. Rabies recombinant plasmids are pRAB-N, pRAB-G, pRAB-NS, pRAB-L and pRAB-M represented by N, G, NS, L and M, respectively. Standard size marker is lamda DNA/*Eco*RI/*Hin*dIII.



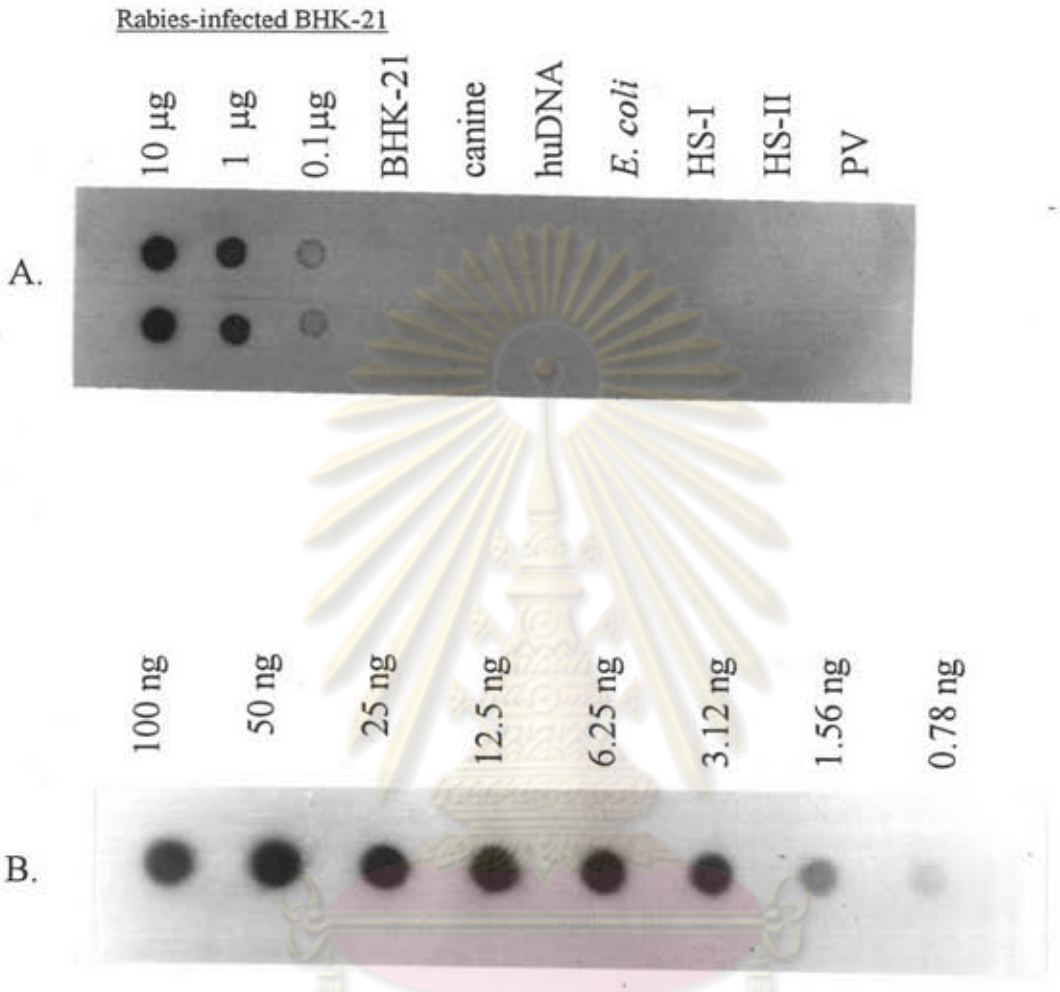


Figure 16. Dot hybridization of rabies viral RNA detected by chemilluminscent-labelled pRAB-NS probe. (A) 10, 1, and 0.1 µg RNA from rabies-infected BHK cells were compared to 5 µg of uninfected BHK-21, canine brain RNA, human DNA (huDNA), *E. coli* DNA, herpes simplex-infected BHK-21 RNA (HS-I and HS-II), and papilloma-infected Vero cells RNA (PV). (B) Self-hybridization of pRAB-NS probe was done in parallel with various amount of PCR product of NS-gene.

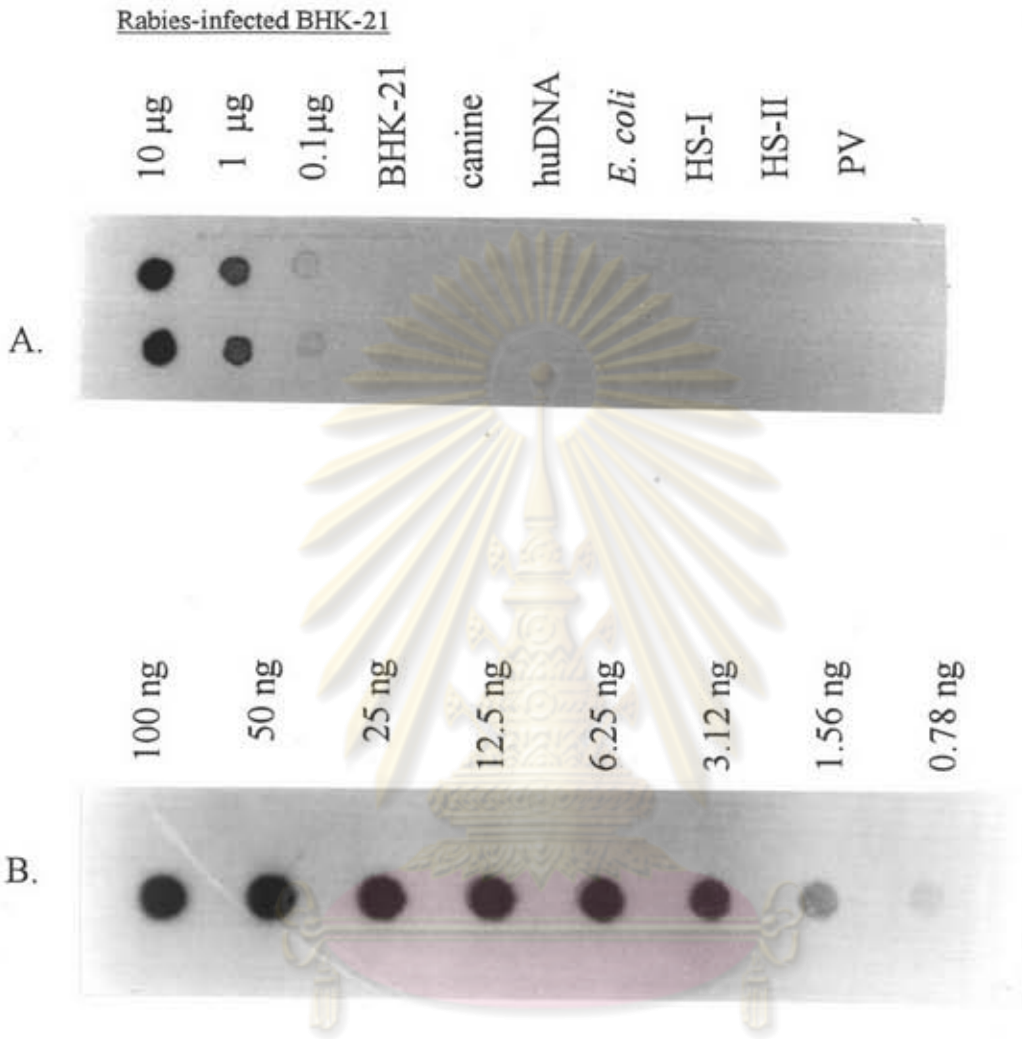


Figure 17. Dot hybridization of rabies viral RNA detected by chemilluminscent-labelled pRAB-L probe. (A) 10, 1, and 0.1 µg RNA from rabies-infected BHK cells were compared to 5 µg of uninfected BHK-21, canine brain RNA, human DNA (huDNA), *E. coli* DNA, herpes simplex-infected BHK-21 RNA (HS-I and HS-II), and papilloma-infected Vero cells RNA (PV). (B) Self-hybridization of pRAB-L probe was done in parallel with various amount of PCR product of L-gene.

detect rabies viral RNA from as little as 100 ng of RNA from infected BHK-21 whereas the pRAB-N, pRAB-G, and pRAB-M probes detected viral RNA from 1 μg of infected BHK-21 RNA (Figure 18, 19 and 20 in picture A).

The probes gave specific signals with RNA from rabies infected cells, and no signal was observed with various non-rabies sources; i.e. *E. coli* DNA, RNA from BHK-21, RNA from herpes virus infected BHK cells, RNA from unifected dog brain as well as human DNA. These results, as shown in Figure 16-20 (picture A), indicated the high specificity of all the five cDNA probes (pRAB-N, pRAB-G, pRAB-NS, pRAB-L and pRAB-M). In addition, labelled pGEM-3Z plasmid was not able to detect RNA from rabies infected cells under tested conditions (data not shown).

A parallel experiment of self hybridization was made. One hundred μg of PCR products from each five structural gene were twofold diluted and spotted to membrane. Hybridization was subsequently carried out with each individual cDNA probes. The results in Figure 16-20 (picture B) showed that pRAB-N, pRAB-G, pRAB-NS, pRAB-L and pRAB-M, was able to detected as low as 1.5, 1.5, 0.2, 0.2 and 0.75 ng of target sequence, respectively.

Application of cDNA Probes in Rabies Diagnosis

In order to assess the potential utilization of rabies cDNA probes in identification of rabies RNA in canine brain samples. RNA of infected and uninfected canine brain samples were prepared and dotted onto membranes. The recombinant plasmids were labelled using ECL random prime system and used as probes. Thirty-nine samples of virus-infected canine brains were tested by dot hybridization technique and compared to routine fluorescent antibody test obtained from Lifestock Promotion Department, Ministry of Agriculture and Cooperation. The results in Figure 21-25 showed the presence of all five rabies RNA species in all 29 samples which had previously been diagnosed as rabid by fluorescent antibody method (FA). Ten negative samples proven by FA and MI were found to be negative by dot hybridization. Neither false positive nor false negative was observed in this study. The labelled plasmid pGEM-3Z failed to detect rabies viral RNA as well as canine brain RNA (data not shown). Thus, the sensitivity and specificity of all five clones was found to be 100% when FA was used as gold standard.

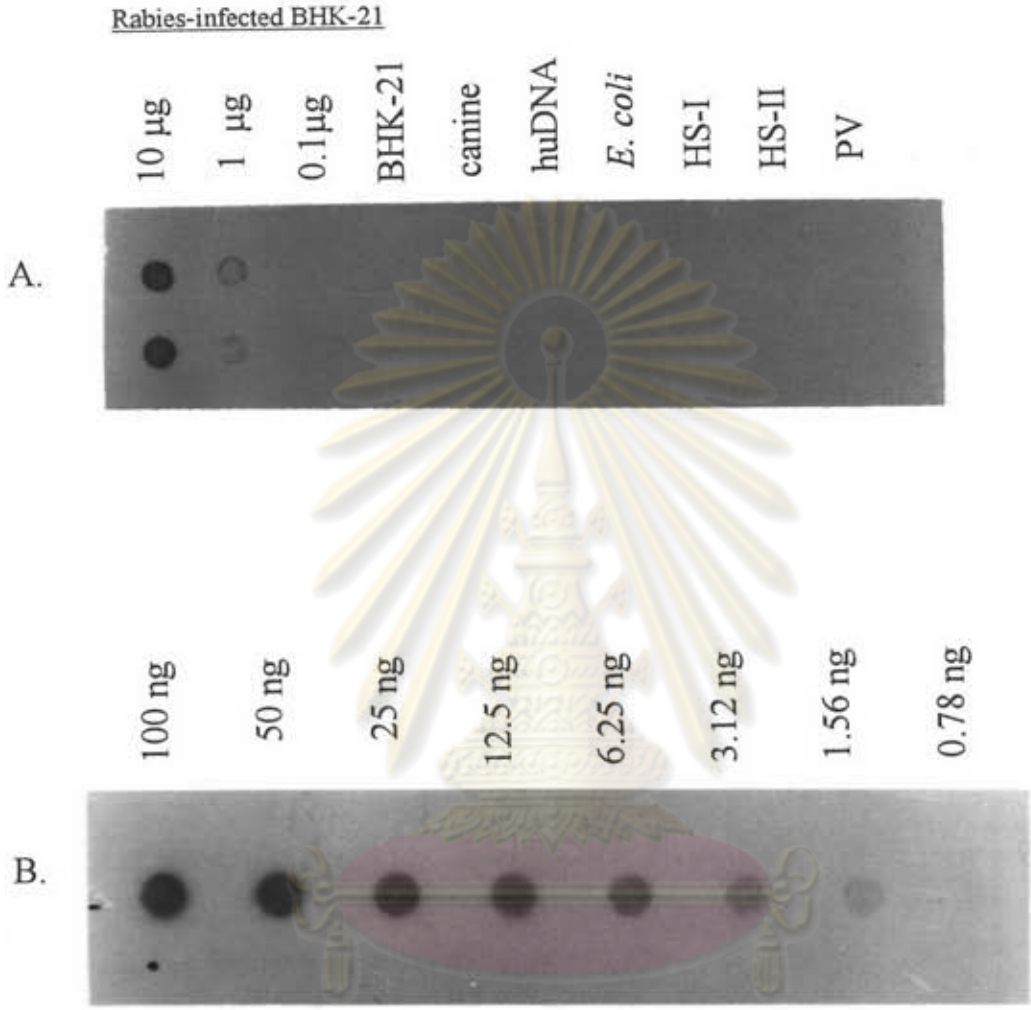


Figure 18. Dot hybridization of rabies viral RNA detected by chemilluminscent-labelled pRAB-N probe. (A) 10, 1, and 0.1 µg RNA from rabies-infected BHK cells were compared to 5 µg of uninfected BHK-21, canine brain RNA, human DNA (huDNA), *E. coli* DNA, herpes simplex-infected BHK-21 RNA (HS-I and HS-II), and papilloma-infected Vero cells RNA (PV). (B) Self-hybridization of pRAB-N probe was done in parallel with various amount of PCR product of N-gene.

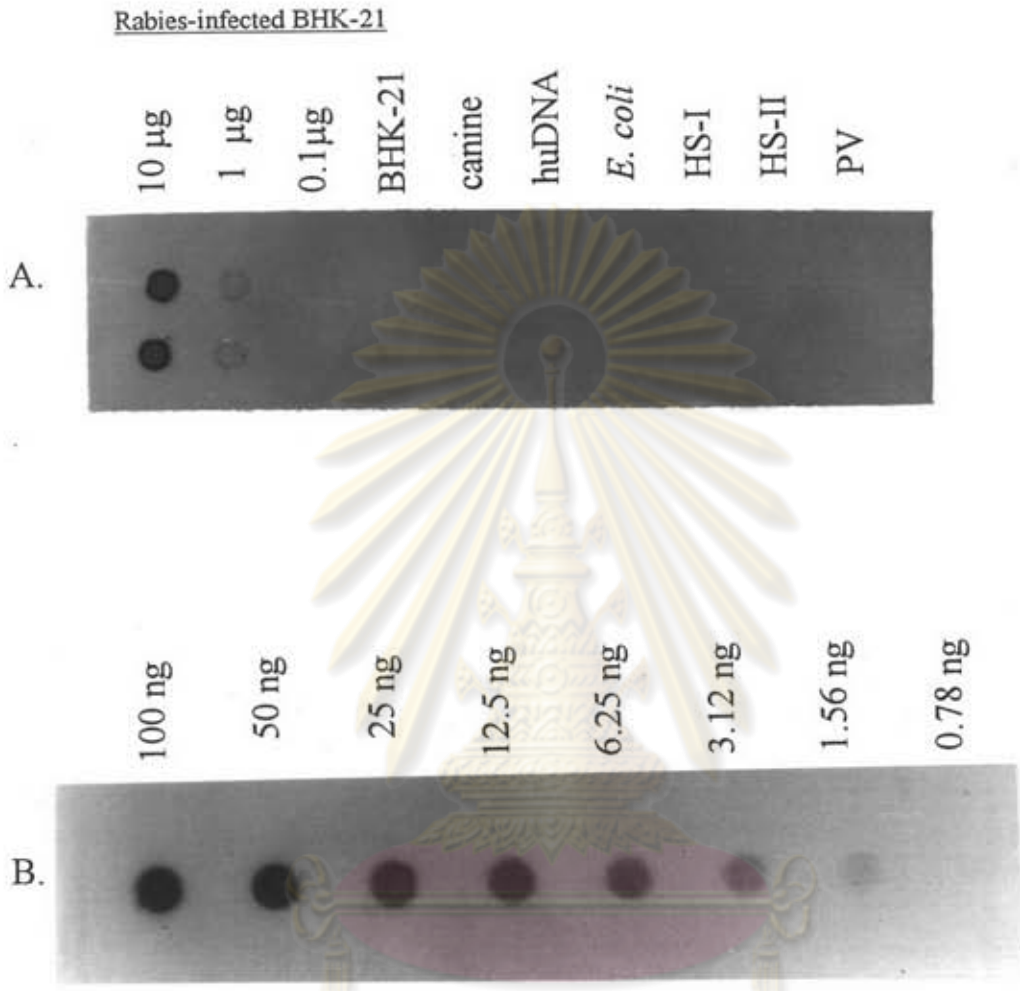


Figure 19. Dot hybridization of rabies viral RNA detected by chemilluminscent-labelled pRAB-G probe. (A) 10, 1, and 0.1 µg RNA from rabies-infected BHK cells were compared to 5 µg of uninfected BHK-21, canine brain RNA, human DNA (huDNA), *E. coli* DNA, herpes simplex-infected BHK-21 RNA (HS-I and HS-II), and papilloma-infected Vero cells RNA (PV). (B) Self-hybridization of pRAB-G probe was done in parallel with various amount of PCR product of G-gene.

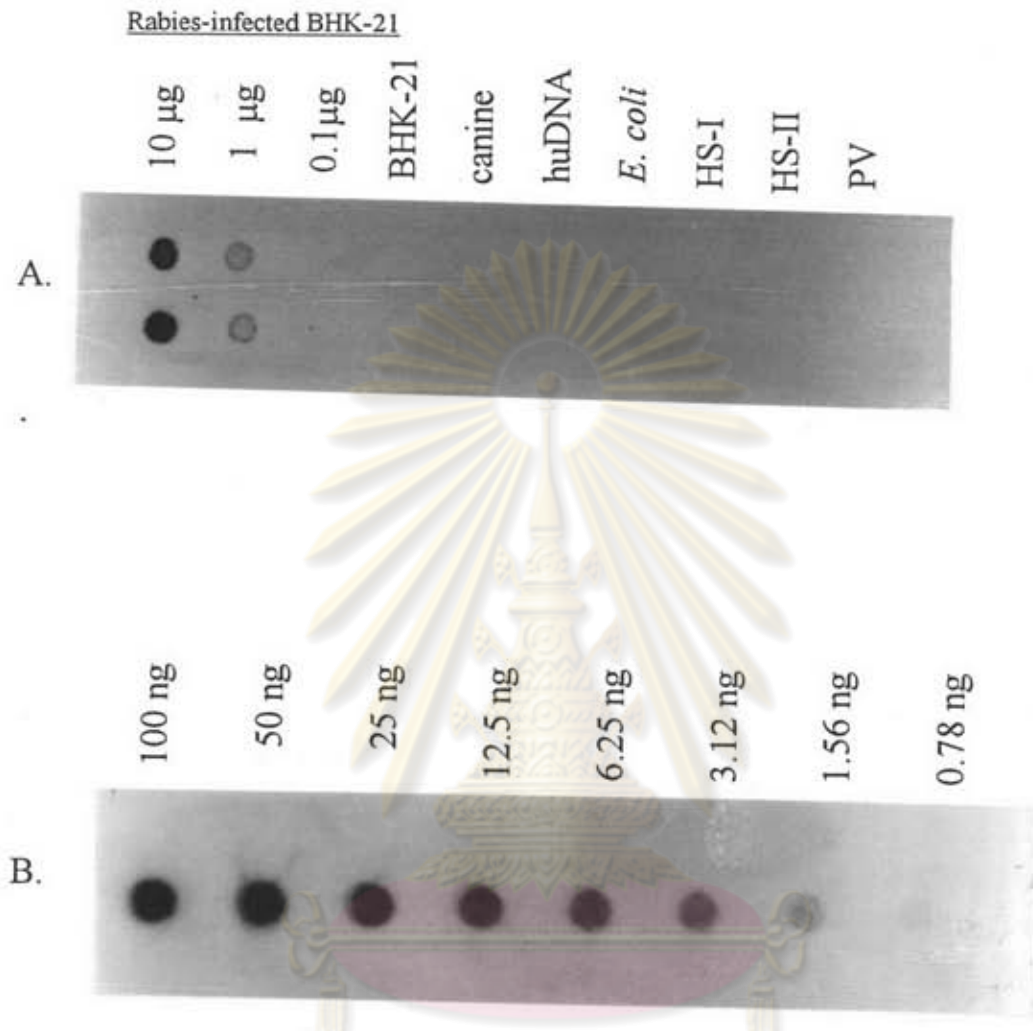


Figure 20. Dot hybridization of rabies viral RNA detected by chemilluminscent-labelled pRAB-M probe. (A) 10, 1, and 0.1 µg RNA from rabies-infected BHK cells were compared to 5 µg of uninfected BHK-21, canine brain RNA, human DNA (huDNA), *E. coli* DNA, herpes simplex-infected BHK-21 RNA (HS-I and HS-II), and papilloma-infected Vero cells RNA (PV). (B) Self-hybridization of pRAB-M probe was done in parallel with various amount of PCR product of M-gene.

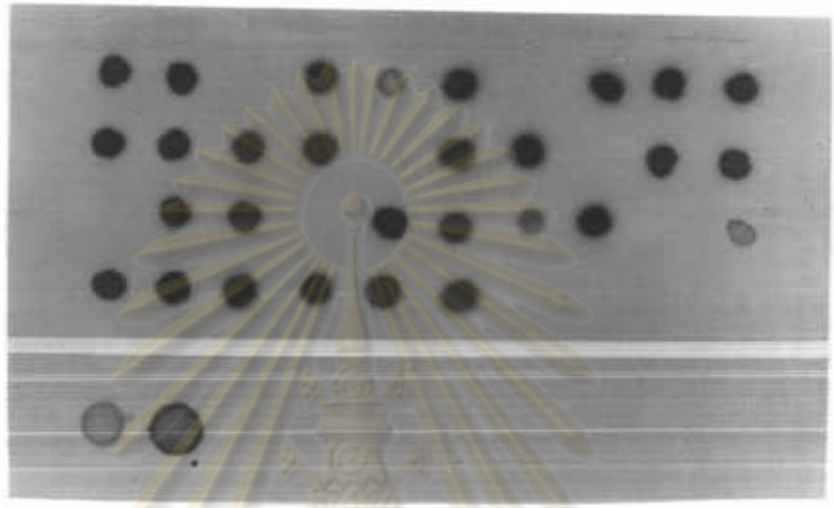


Figure 21. Dot-hybridization of RNA extract from canine brain samples detected by chemilluminescent-labelled pRAB-N probe. Each dots contained 10 μg of RNA.

+	+	-	+	+	+	-	+	+	+
+	+	+	+	-	+	+	-	+	+
-	+	+	-	+	+	+	+	-	+
+	+	+	+	+	+	-	-	-	
$+^1$	$+^2$	$-^1$	$-^2$						

+ = rabies-infected brain, - = uninfected brain, $+^1$ = self-hybridization with PCR product, $+^2$ = rabies-infected BHK-21, $-^1$ = PCR amplified uninfected BHK-21, $-^2$ = uninfected BHK-21

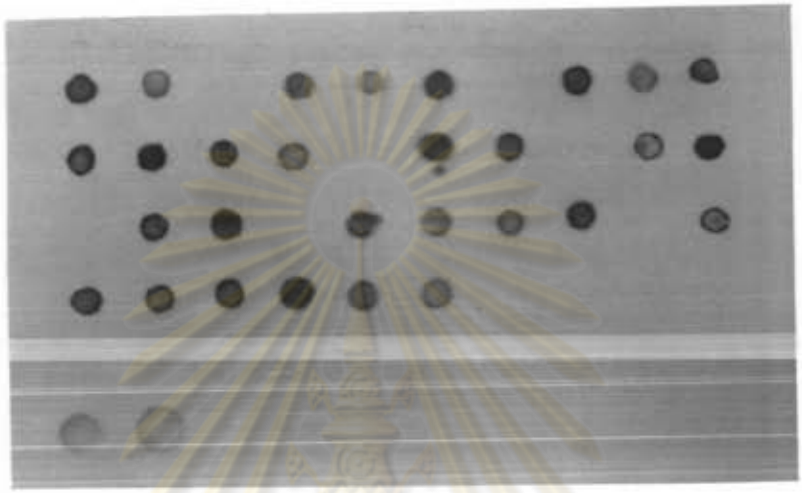


Figure 22. Dot-hybridization of RNA extract from canine brain samples detected by chemilluminescent-labelled pRAB-G probe. Each dots contained 10 μg of RNA.

+	+	-	+	+	+	-	+	+	+
+	+	+	+	-	+	+	-	+	+
-	+	+	-	+	+	+	+	-	+
+	+	+	+	+	+	-	-	-	
$+^1$	$+^2$	$-^1$	$-^2$						

+ = rabies-infected brain, - = uninfected brain, $+^1$ = self-hybridization with PCR product, $+^2$ = rabies-infected BHK-21, $-^1$ = PCR amplified uninfected BHK-21, $-^2$ = uninfected BHK-21

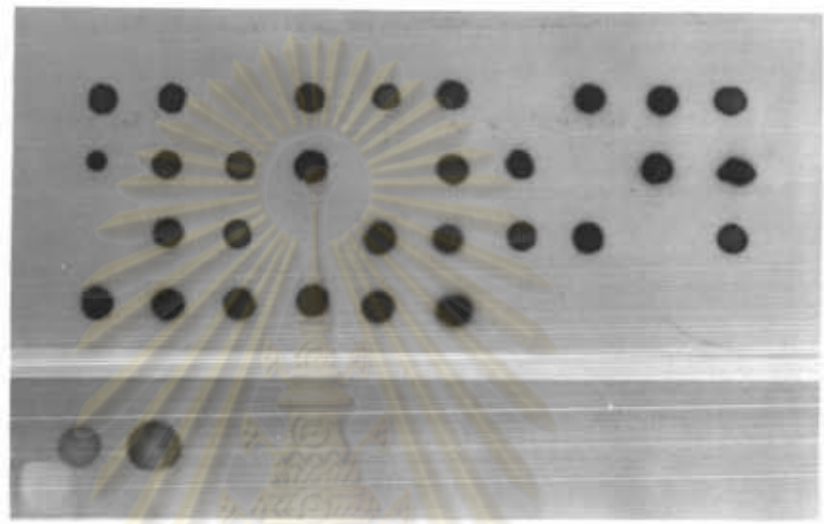


Figure 23. Dot-hybridization of RNA extract from canine brain samples detected by chemilluminescent-labelled pRAB-NS probe. Each dots contained 10 μg of RNA.

+	+	-	+	+	+	-	+	+	+
+	+	+	+	-	+	+	-	+	+
-	+	+	-	+	+	+	+	-	+
+	+	+	+	+	+	-	-	-	
$+^1$	$+^2$	$-^1$	$-^2$						

+ = rabies-infected brain, - = uninfected brain, $+^1$ = self-hybridization with PCR product, $+^2$ = rabies-infected BHK-21, $-^1$ = PCR amplified uninfected BHK-21, $-^2$ = uninfected BHK-21

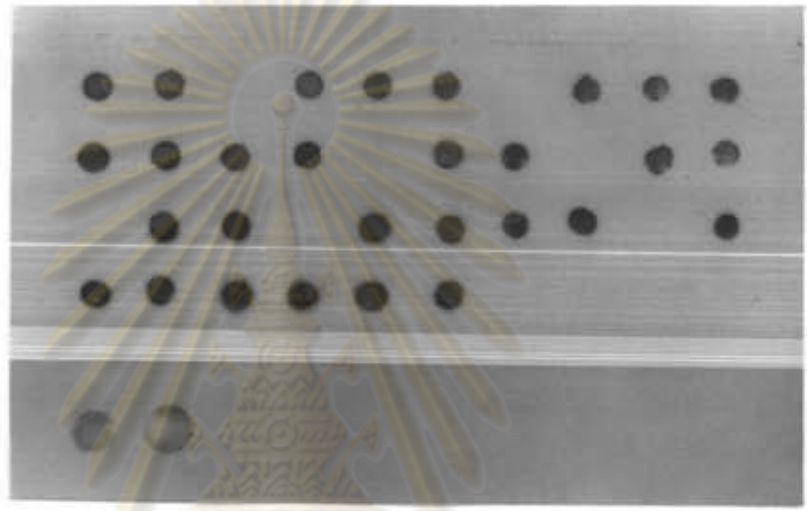


Figure 24. Dot-hybridization of RNA extract from canine brain samples detected by chemilluminescent-labelled pRAB-M probe. Each dots contained 10 μg of RNA.

+	+	-	+	+	+	-	+	+	+
+	+	+	+	-	+	+	-	+	+
-	+	+	-	+	+	+	+	-	+
+	+	+	+	+	+	-	-	-	
$+^1$	$+^2$	$-^1$	$-^2$						

+ = rabies-infected brain, - = uninfected brain, $+^1$ = self-hybridization with PCR product, $+^2$ = rabies-infected BHK-21, $-^1$ = PCR amplified uninfected BHK-21, $-^2$ = uninfected BHK-21

Figure 25. Dot-hybridization of RNA extract from canine brain samples detected by chemilluminescent-labelled pRAB-L probe. Each dots contained 10 μg of RNA.

+	+	-	+	+	+	-	+	+	+
+	+	+	+	-	+	+	-	+	+
-	+	+	-	+	+	+	+	-	+
+	+	+	+	+	+	-	-	-	
$+^1$	$+^2$	$-^1$	$-^2$						

+ = rabies-infected brain, - = uninfected brain, $+^1$ = self-hybridization with PCR product, $+^2$ = rabies-infected BHK-21, $-^1$ = PCR amplified uninfected BHK-21, $-^2$ = uninfected BHK-21

Synchronization of BHK-21 Cells

BHK-21 cells were synchrony induced by releasing from serum starvation. The stationary phase cells were incubated in minimal medium without supplementation of glutamine and serum for 6 hrs. The cells were released by replacement with fresh complete medium. Induced cells were pulse-labelled with ^{3}H-uridine into newly synthesized RNA. Cells were then precipitated with cold TCA and scintillation counted. Details of method are described in Appendix C. Rate of RNA synthesis was determined by measuring the incorporation of ^{3}H-uridine into RNA. The rate of cellular RNA synthesis was reduced during maintenance of cells in starvation (data not shown). At time interval after the replacement of fresh complete medium, the rate of cellular RNA synthesis was twofold increased and reached stationary within 4 hrs (Figure 26) whereas the number of cells was slowly increased, however, and doubled within 18 hrs. Increasing rate of RNA synthesis indicated that cells were induced to resume the traverse of the life cycle in synchrony and were capable of macromolecule synthesis without delay.

Characterization of the Transcription of Rabies Virus

The transcriptional events of rabies virus was determined by following the transcript products at various postinfection periods. The RNA-free viral seed prepared as described in chapter 3, was adsorbed to synchronized BHK-21 monolayer in tissue culture flasks. Aliquots of cells were collected periodically and lysed by boiling. Cell lysate were collected and treated with proteinase K. Transcripts were reversed transcribed by oligo(dT) and subjected to PCR. Amplification of each rabies gene product was extended by primer pairs in Table 2. Details of method were described in chapter 3. Amplification products were analyzed on 1.2% agarose gel electrophoresis. In Figure 27, the RNA isolated from 0-7 hr postinfection (hpi) was individually detected for five species of rabies transcript by PCR technique. No rabies transcripts were detectable at $t = 0$. This indicates that contamination of RNA transcripts in viral seed was successfully removed. Thus the appearance of amplified gene products was obtained from newly synthesized transcripts. The N-transcripts was detected within 1 hpi. The NS-, M- G-, and L-transcripts sequentially appeared as ethidium bromide stained bands on agrase gel at 3, 4, 5 and 2 hpi, respectively. All five species of rabies mRNA transcripts were detected within 5 hrs after infection. The transcriptional events of rabies virus in infected BHK-21 cells by RT-PCR detection appeared to preceed in the order of N, L, NS, M, and G.

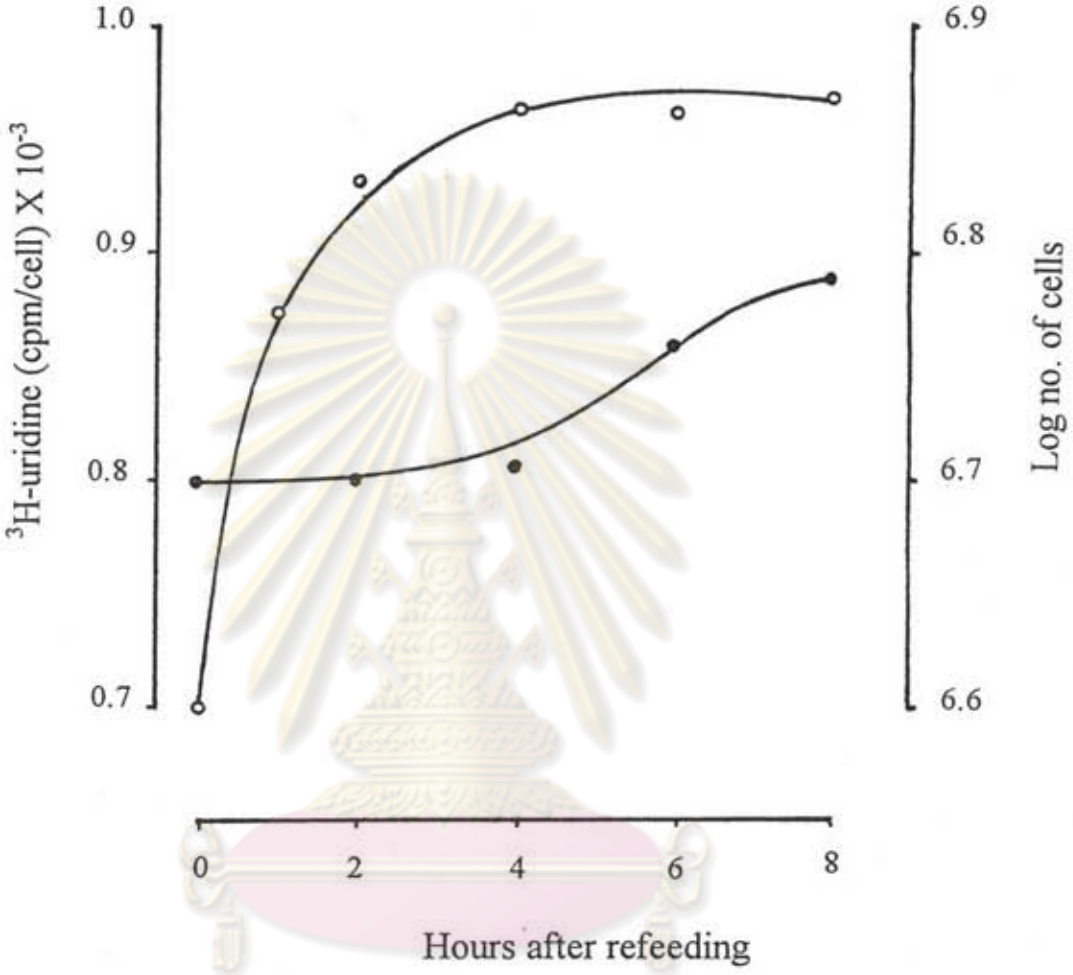


Figure 26. Rate of ^{3}H-uridine incorporation into BHK-21 cells. Synchronized BHK-21 cells were induced by refeeding with fresh complete medium at t = 0. At time interval thereafter, aliquot of cell suspension were incubated with ^{3}H-uridine. Cells were harvested and then precipitated with cold TCA.

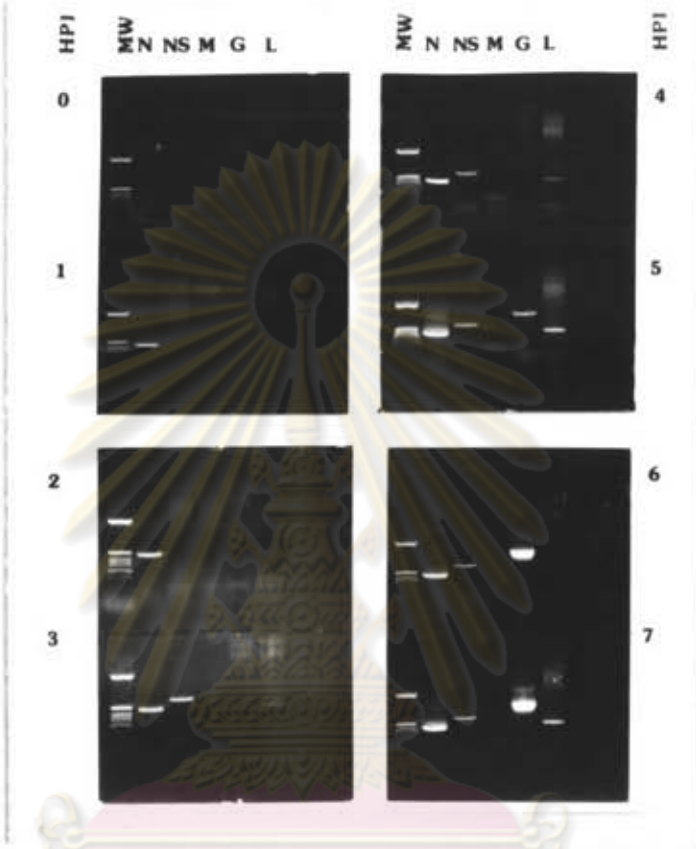


Figure 27. Transcriptional events of rabies viral genes characterized by RNA-PCR technique. Whole cell lysates harvested from rabies-infected BHK-21 at 0-6 hr postinfection (hpi) were subjected to first-stranded cDNA synthesis by oligo(dT) extension. Amplification of specific genes was performed in reaction mixtures containing sequence specific primer pairs. Agarose-gel is shown. Lane MW is size marker, pBR322/*Hin*fI.

In our previous experiments, transcription of rabies virus were carried out in non-synchronized BHK-21 monolayer. Many trials were obtained to examine the transcripts at various hr after infection (from 0-12 hpi). However, the presence of viral transcripts as well as the order of transcription were not reproducible within 3 hpi. The results suggested the existence of some variations in cell culture. One of the most critical drawback is the variable stages of cell cycle. Under starvation conditions, the entire population of BHK-21 could be made to stop in the G1 phase. On subsequent refeeding with fresh medium, these cells synthesized RNA as well as DNA and divided synchronously. When transcription of rabies virus was observed in synchronized BHK-21, the reproducibility was improved and appropriate time interval was developed. Furthermore, the starved cells should be maintaned in fresh medium for at least 6 hrs. In our experience, brief incubation in fresh medium resulted at least six-hour shift in viral RNA synthesis.

After many trials, transcription of N-gene was detected as earlier as 60 min after postinfection. The synthesis of N transcript was increased in first 5 hpi and the accumulation appeared on ethidium bromide-stained gel was decreased at 6 hpi (Figure 27) suggesting the degradation of N transcript. The RNA synthesis of L-gene was detected at 2 hpi. Accumulation of L-transcript was decreased at 6 hpi and raised up again at 7 hpi. The NS-transcript was found before 3 hpi and maintained at low concentration whereas the M-transcript was appeared at 3 hpi and maintained at low concentration as NS-transcript. The G-transcript was the last transcription product detected at 5 hpi and accumulated at high concentration.