CHAPTER IV

RESULTS

4.1 *Leptospira* 16S rRNA detection

Leptospira 16S rRNA detection was done to demonstrate the presence of *Leptospira*. No 16S rRNA was detected in cDNA extracts from kidneys and livers of uninfected hamsters (Figure 3A and Figure 3B). A 290-bp PCR product of 16S rRNA gene was detected in all kidney (Figure 4) and liver (Figure 5) tissues from hamsters infected with pathogenic *Leptospira*. This suggested that *Leptospira* could be found in kidneys and livers on or before day 3 postinfection and the organisms could still be detected on day 7.

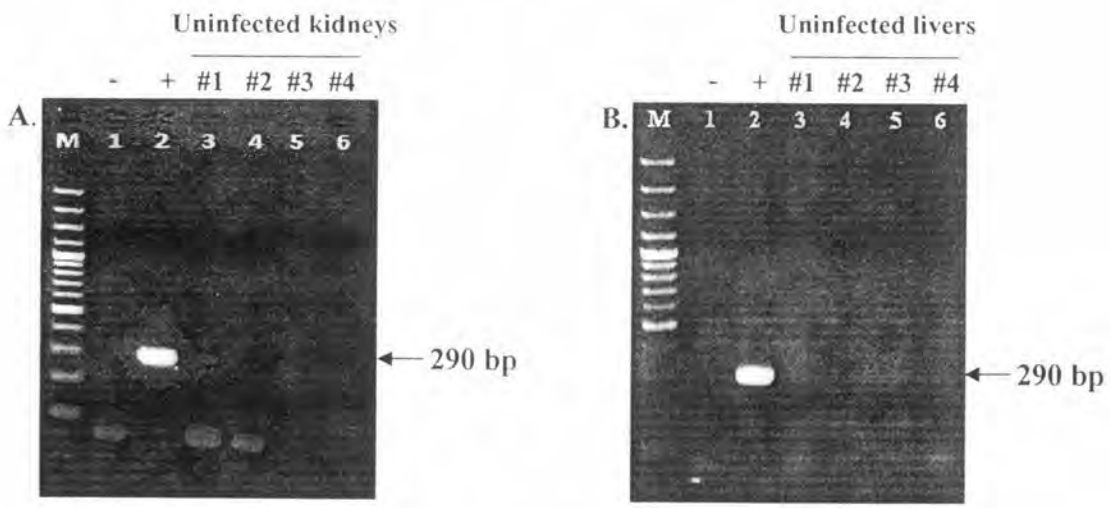


Figure 3 16S rRNA expression in kidneys and livers of uninfected hamsters. cDNA was transcribed from RNA extracted from uninfected kidneys (3A) and livers (3B) of hamsters and PCR using 16S rRNA primers was performed as described in Materials and Methods. Lane M, 100-bp marker; Lane 1, negative control; Lane 2, positive control; Lane 3, 4, 5, and 6 uninfected kidneys and livers from four hamsters.

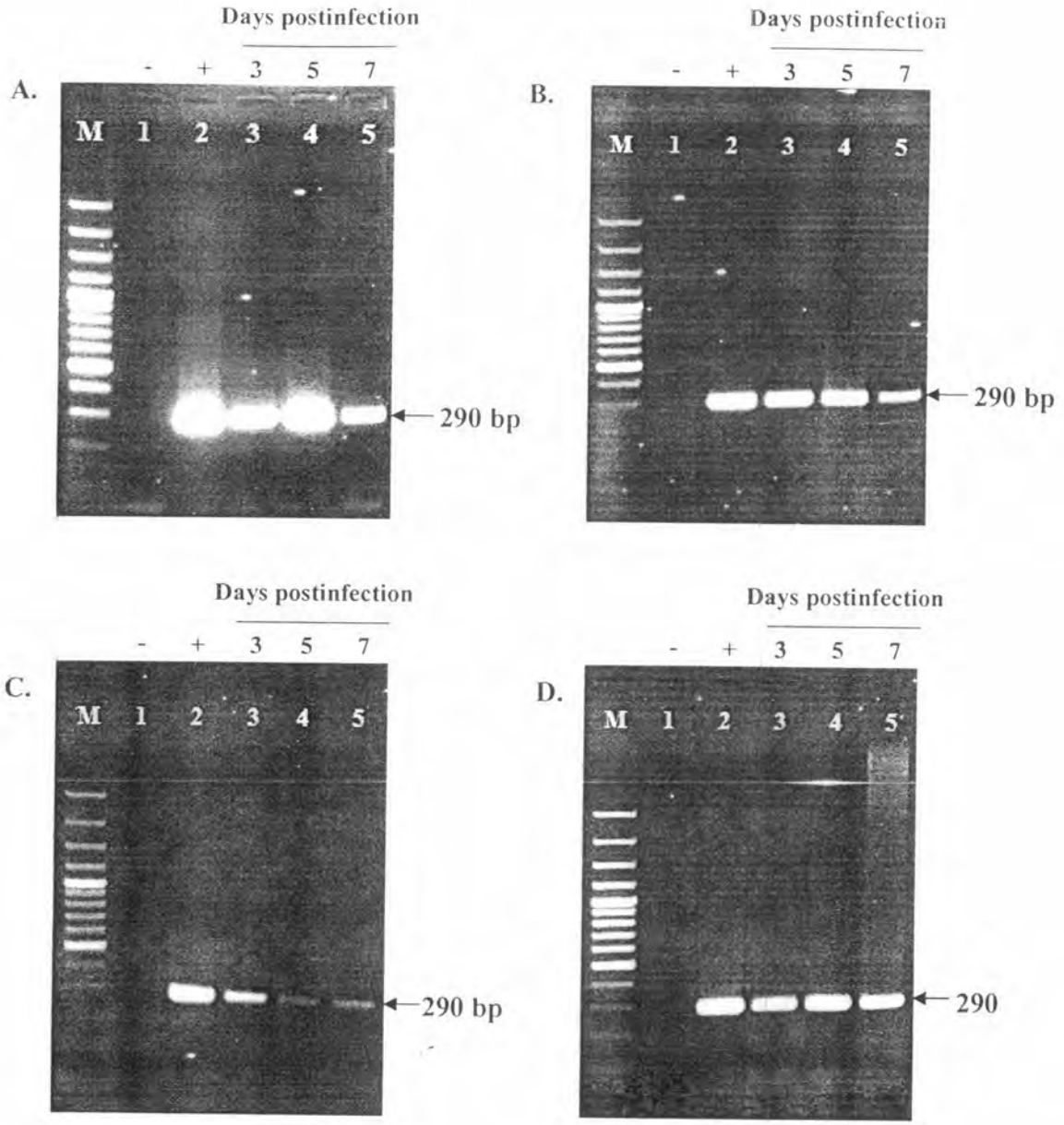


Figure 4 16S rRNA expression in kidneys of hamsters infected with pathogenic *Leptospira*. Hamsters infected with pathogenic *Leptospira* were sacrificed on day 3, 5 and 7 post infections. Four hamsters were used for each timepoint. RNA was extracted from kidney tissues and RT-PCR was performed for 16S rRNA expression. LaneM ,100-bp marker; Lane 1, negative control; Lane 2, positive control; Lanes 3, 4, and 5, kidneys from hamsters infected with pathogenic *Leptospira* at 3, 5, 7 days post infection, respectively. The data demonstrated were from 4 replicates.

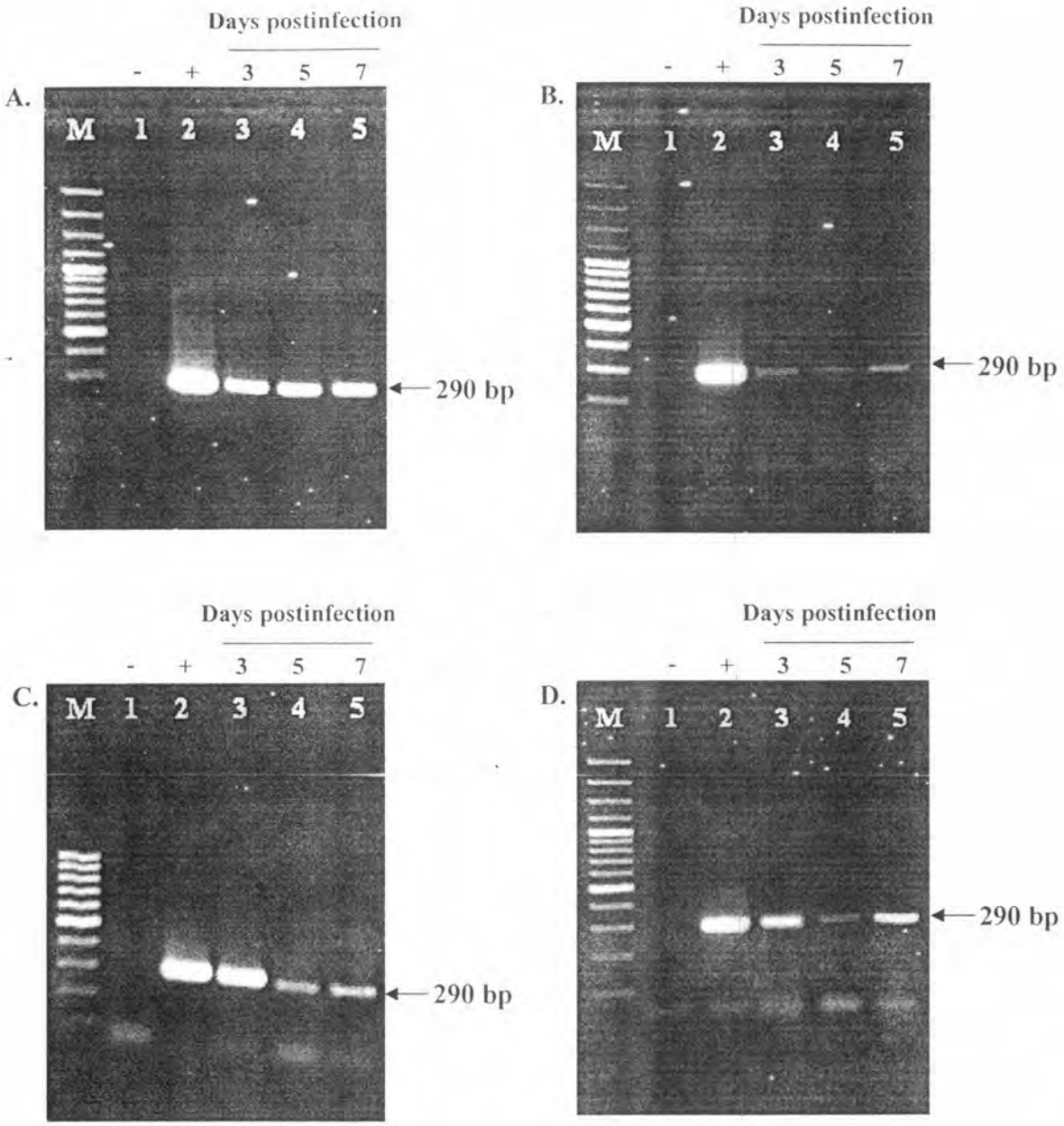


Figure 5 16S rRNA expression in livers of hamsters infected with pathogenic *Leptospira*. Hamsters infected with pathogenic *Leptospira* were sacrificed on day 3, 5 and 7 post infections. Four hamsters were used for each timepoint. RNA was extracted from liver tissues and RT-PCR was performed for 16S rRNA expression. Lane M, 100-bp marker; Lane 1, negative control; Lane 2, positive control; Lanes 3, 4, and 5, livers from hamsters infected with pathogenic *Leptospira* at 3, 5, 7 days post infection, respectively. The data demonstrated were from 4 replicates.

4.2 LipL32 mRNA expression in kidneys and livers

Since LipL32 is the most abundant *Leptospira* OMPs and its roles as a vaccine candidate, an antigen for serological diagnosis and a virulence factor have been studied, detection of LipL32 expression in kidneys and livers of infected hamsters was investigated. LipL32 expression could not be detected by regular PCR so nested PCR was performed. The 506-bp nested PCR product indicated the presence of LipL32 mRNA expression. There was no LipL32 gene expression detected in RNA extracted from kidneys and livers of uninfected hamsters (Figure 6) whereas the expression was observed in all kidney (Figure 7) and liver (Figure 8) tissues from hamsters infected with pathogenic *Leptospira*.

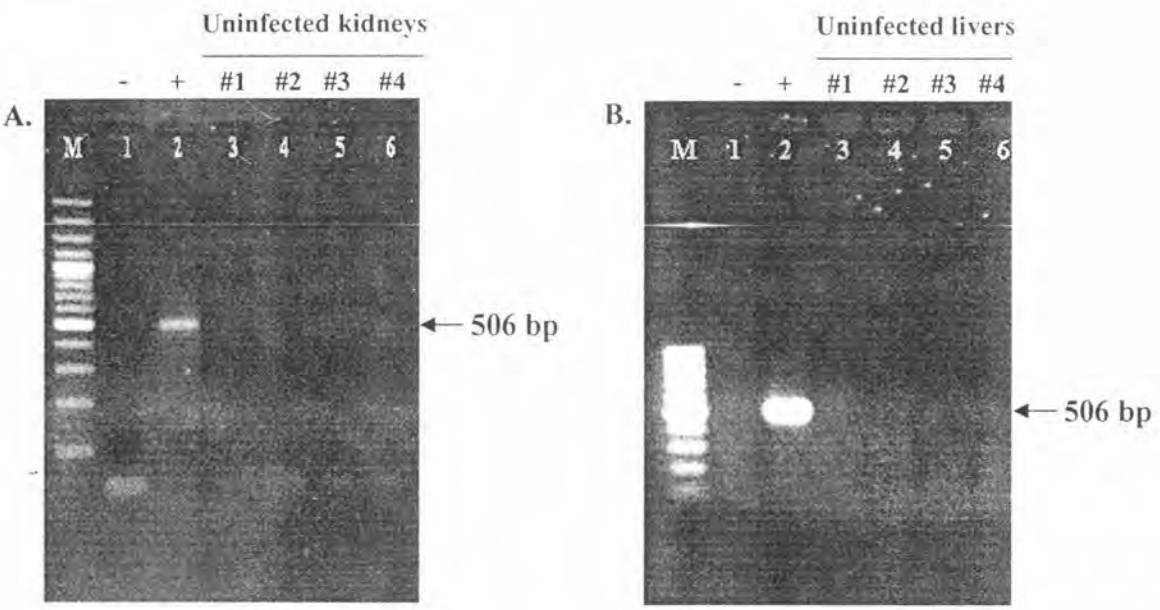


Figure 6 Detection of LipL32 gene expressions in uninfected kidneys and livers. cDNA was transcribed from RNA extracted from uninfected kidney (6A) and livers (6B) of hamsters and PCR using LipL32 primers was performed as described in Materials and Methods. Lane M, 100-bp marker; Lane 1, negative control; Lane 2, positive control; Lane 3, 4, 5, and 6, kidneys and livers from four uninfected hamsters.

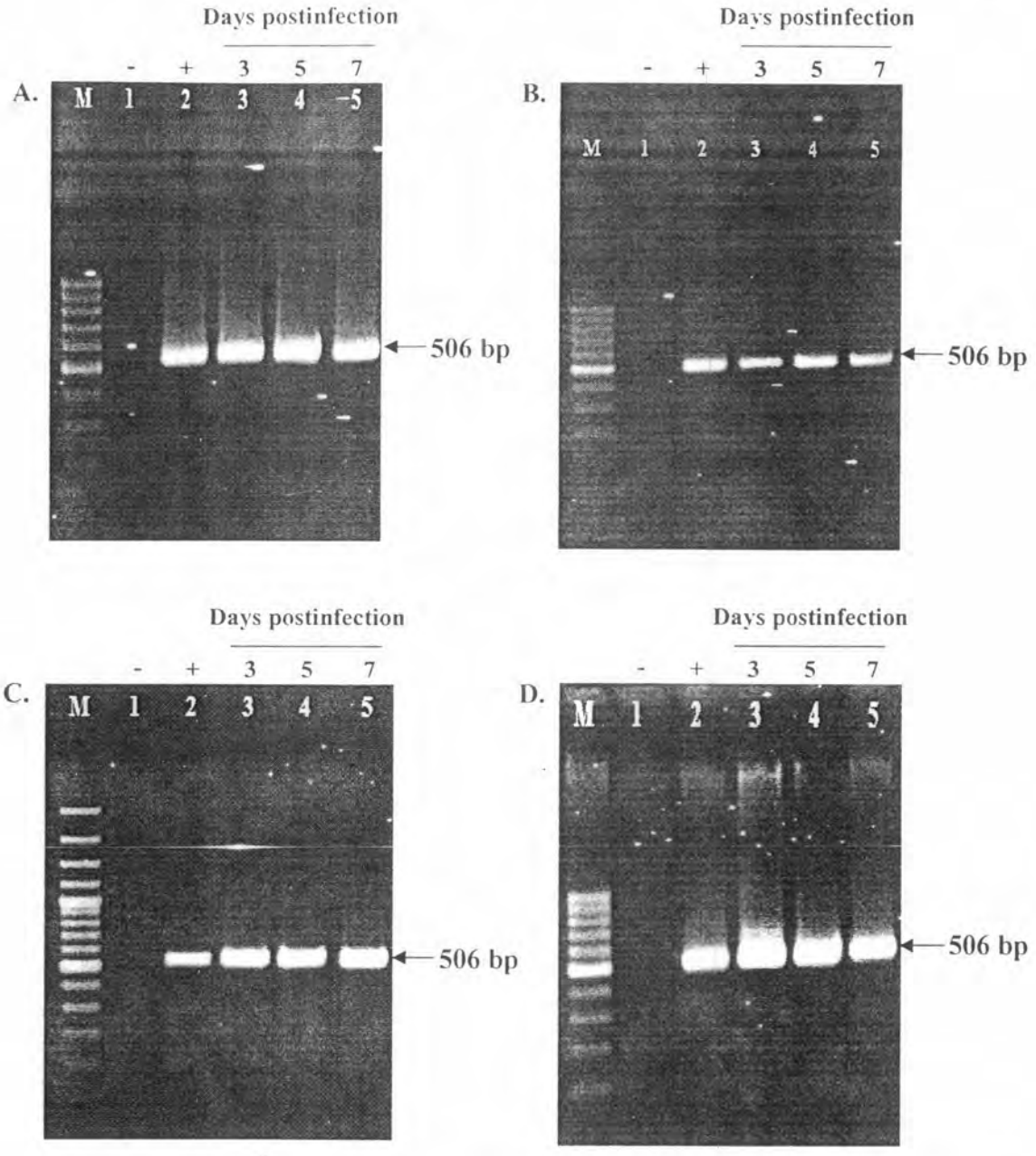


Figure 7 LipL32 gene expression in kidneys of hamsters infected with pathogenic *Leptospira*. Hamsters infected with pathogenic *Leptospira* were sacrificed at day 3, 5 and 7 postinfection. RNA was extracted from kidney tissues and RT-PCR was performed to demonstrate LipL32 gene expression. Lane M, 100-bp marker; Lane 1, negative control; Lane 2, positive control; Lanes 3, 4, and 5, kidneys from hamsters infected by pathogenic *Leptospira* at 3, 5, 7 days post infection, respectively. The data demonstrated were from 4 replications.

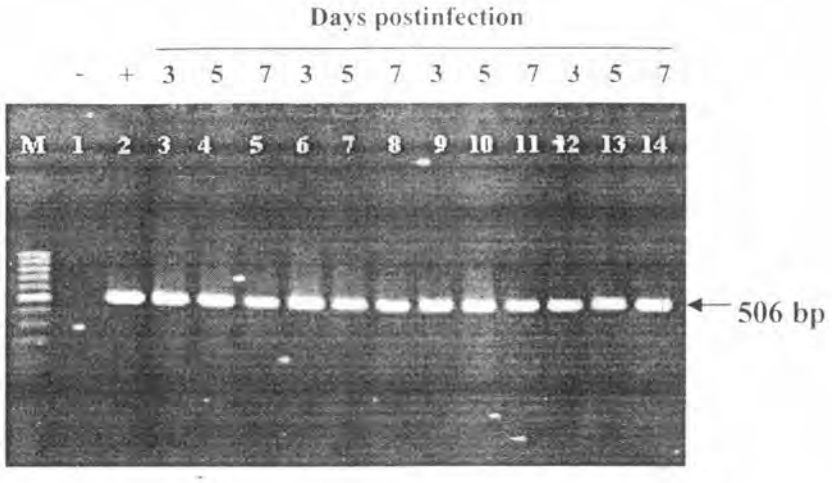


Figure 8 LipL32 gene expression in livers of hamsters infected with pathogenic *Leptospira*. Hamsters infected with pathogenic *Leptospira* were sacrificed at day 3, 5 and 7 postinfection. RNA was extracted from liver tissues and RT-PCR was performed to demonstrate LipL32 expression gene. Lane M, 100-bp marker; Lane 1 : negative control, Lane 2 : positive control, Lane 3, 4, 5 : liver infected by pathogenic *Leptospira* at 3, 5, 7 days post infection in the first replication, Lane 6, 7, 8 : liver infected by pathogenic *Leptospira* at 3, 5, 7 days post infection in the second replication, Lane 9, 10, 11 : liver infected by pathogenic *Leptospira* at 3, 5, 7 days post infection in the third replication, Lane 12, 13, 14 : liver infected by pathogenic *Leptospira* at 3, 5, 7 days post infection in the last replication.

4.3 HPRT, TNF-α, TGF-β, IL-10 and IP-10 mRNA expression

4.3.1 HPRT mRNA expression

RT-PCR for HPRT expression was done to demonstrate successful RNA extraction. The 206-bp PCR product suggesting HPRT gene expression was detected in kidneys and livers from both uninfected and *Leptopsira* infected hamsters (Figures 9-11).

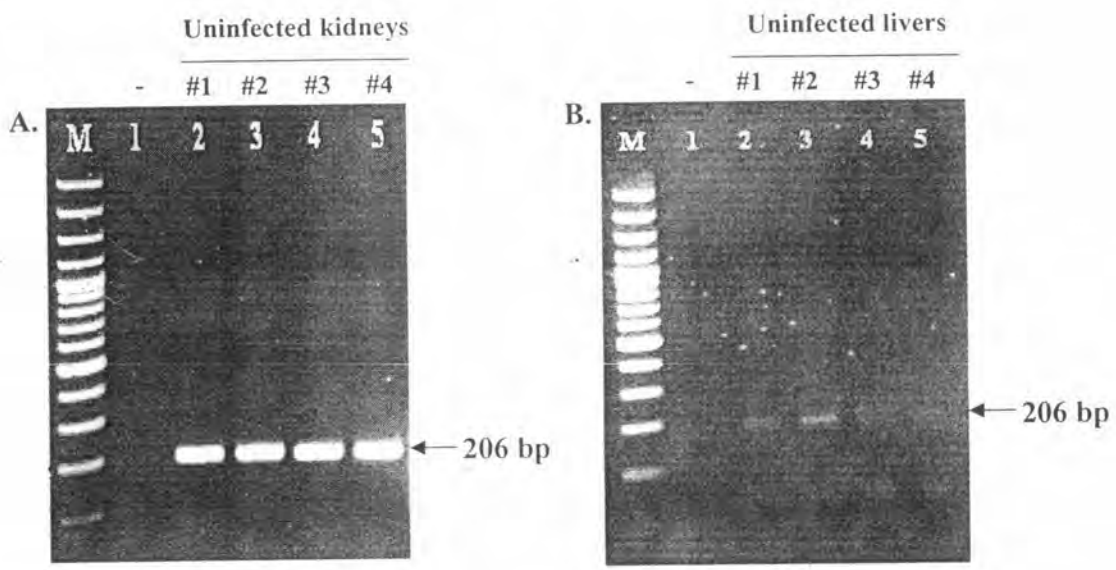


Figure 9 HPRT mRNA expression in kidneys and livers of uninfected hamsters. cDNA was transcribed from RNA extracted from kidney (9A) and livers (9B) of uninfected hamsters and PCR using HPRT primers was performed as described in Materials and Methods. Lane M, 100-bp marker; Lane 1, negative control; Lanes 2, 3, 4, and 5, kidneys and livers from four uninfected hamsters.

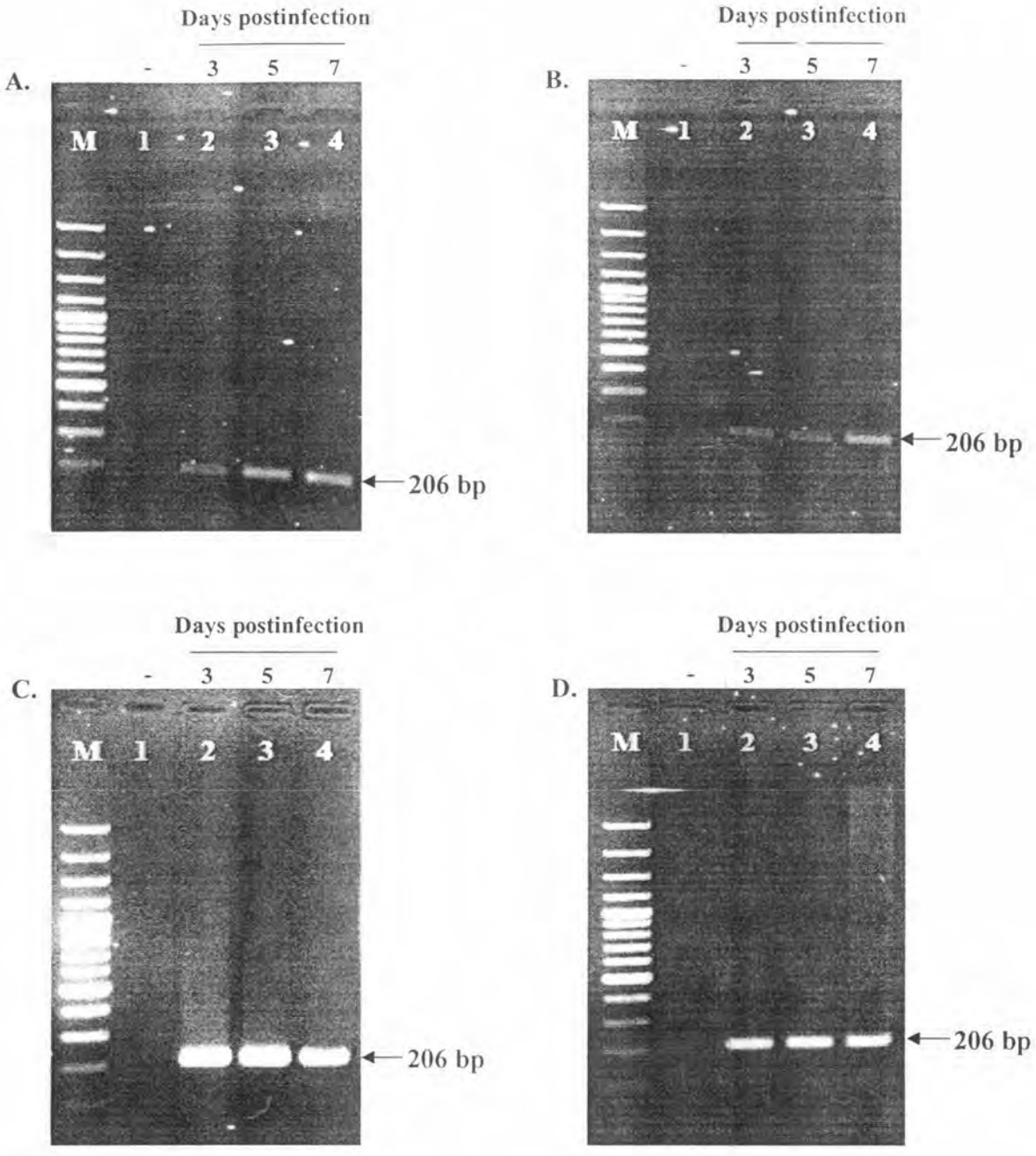


Figure 10 HPRT mRNA expression in kidneys from hamsters infected with pathogenic *Leptospira*. Hamsters infected with pathogenic *Leptospira* were sacrificed at day 3, 5 and 7 post infection. RNA was extracted from kidney tissues and RT-PCR was performed for HPRT expression. Lane M, 100-bp marker, Lane 1, negative control; Lane 2, 3, and 4, kidneys from hamsters infected with pathogenic *Leptospira* at 3, 5, 7 days post infection, respectively. The data demonstrated were from 4 replicates.

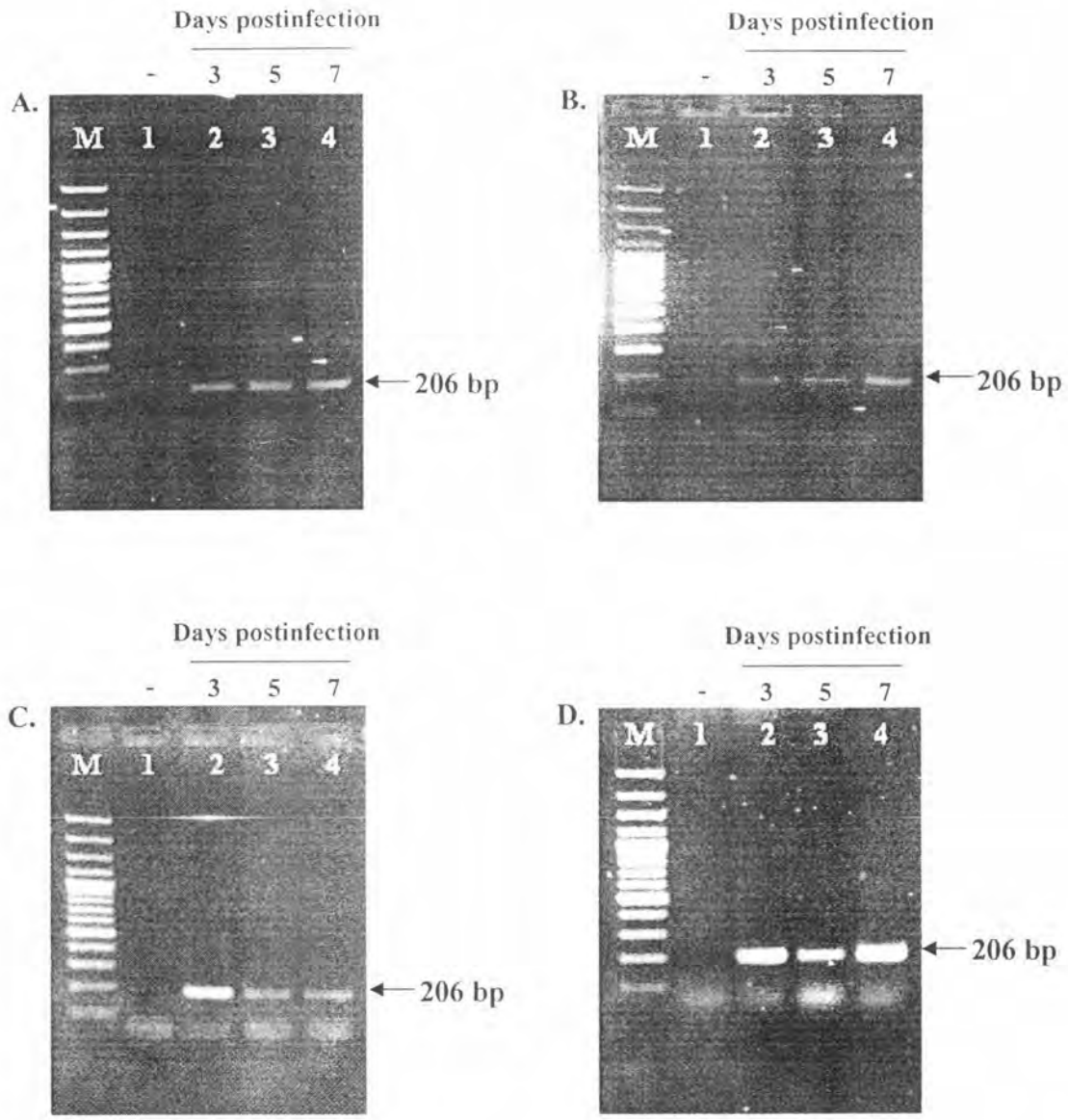


Figure 11 HPRT mRNA expression in livers of hamsters infected with pathogenic *Leptospira*. Hamsters infected with pathogenic *Leptospira* were sacrificed at day 3, 5 and 7 post infection. RNA was extracted from liver tissues and RT-PCR was performed for HPRT expression. Lane M, 100-bp marker; Lane 1, negative control; Lanes 2, 3, and 4, livers from hamsters infected with pathogenic *Leptospira* at 3, 5, 7 days post infection, respectively. The data demonstrated were from 4 replicates.

4.3.2 TNF-α mRNA expression

The 126-bp PCR product indicated the presence of TNF-α mRNA gene expression. TNF-α mRNA expression was detected in both kidney and liver tissues from hamsters infected with pathogenic *Leptospira* (Figures 13-14). The expression was observed since day 3 post-infection. However, TNF-α gene expression was also detected in all kidney tissues from uninfected hamsters.

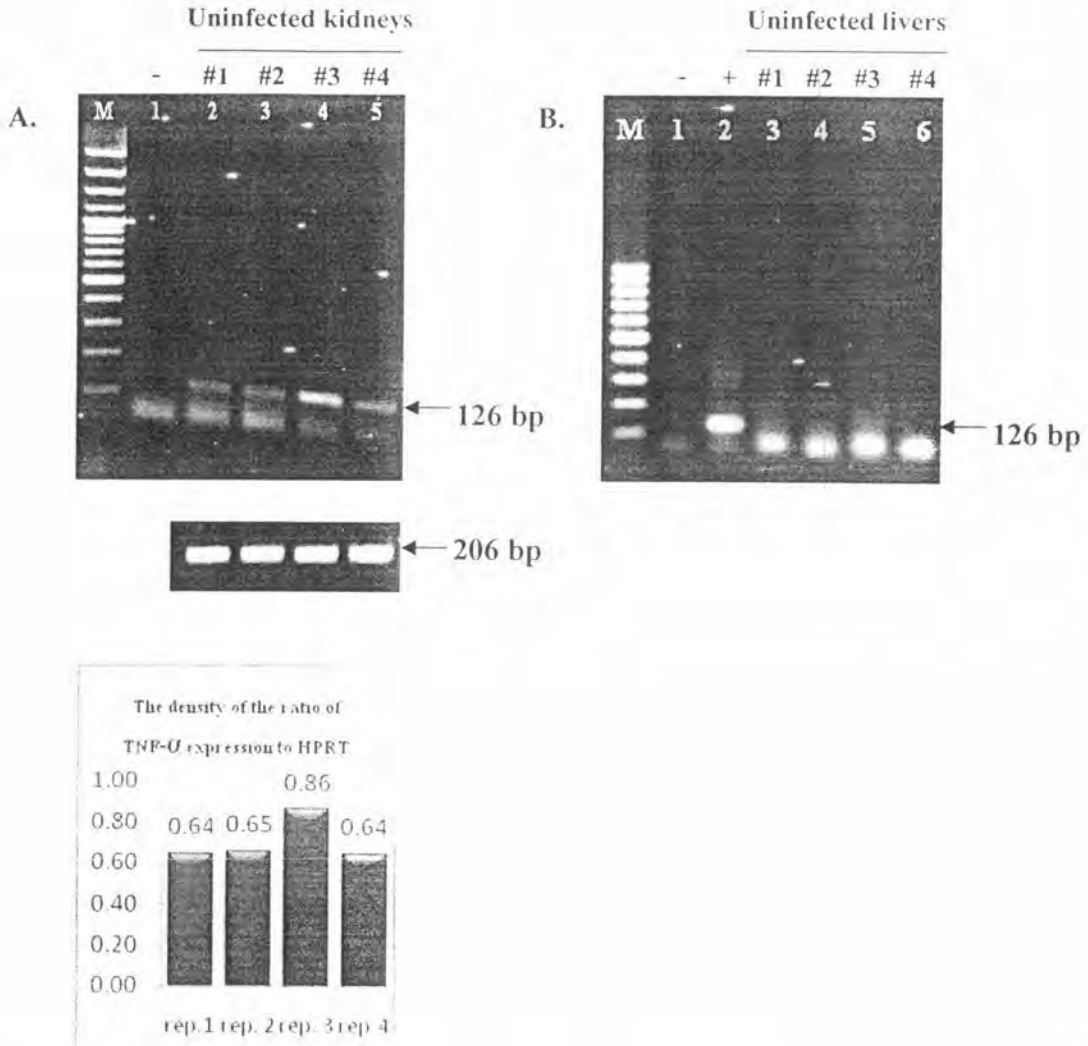
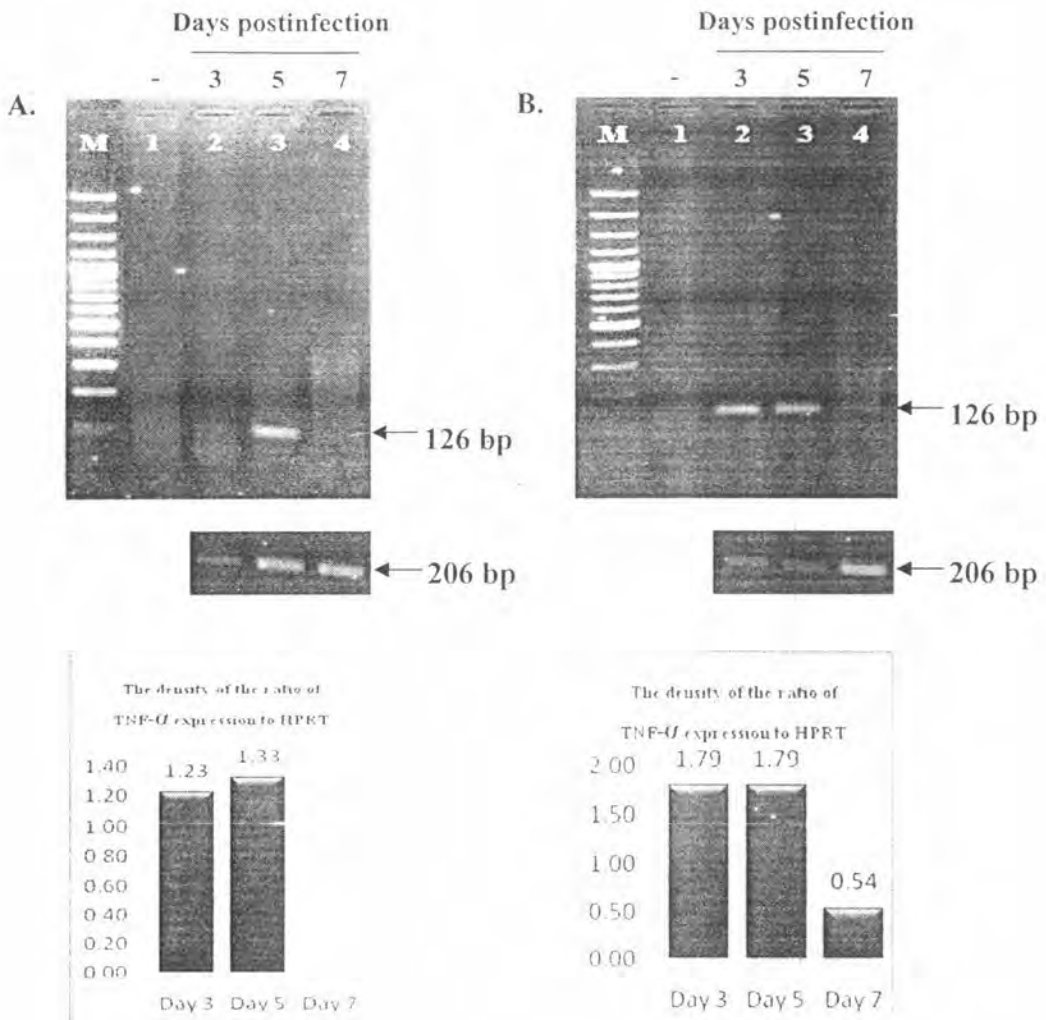


Figure 12 TNF-α expression in kidneys and livers of uninfected hamsters. cDNA was transcribed from RNA extracted from kidneys (12A) and livers (12B) of uninfected hamsters and PCR using TNF-α primers was performed as described in Materials and Methods. In Figure 12A, Lane M, 100-bp marker; Lane 1, negative control; Lanes 2, 3, 4, and 5, kidneys from four uninfected hamsters. In Figure 12B, Lane M, 100-bp marker; Lane 1, negative control; Lane 2, positive control; Lane 3, 4, 5, and 6, livers from four uninfected hamsters. The lower figure showed HPRT mRNA expression in each sample and the graph underneath Figure 12A, demonstrates density ratio between bands from TNF-α and HPRT PCR products.


Days postinfection
- 3 5 7
A.
M 1 2 3 4
126 bp
206 bp
Days postinfection
- 3 5 7
B.
M 1 2 3 4
126 bp
206 bp
The density of the ratio of
TNF-α expression to HPRT
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
1.23
1.33
Day 3 Day 5 Day 7
The density of the ratio of
TNF-α expression to HPRT
2.00
1.50
1.00
0.50
0.00
1.79
1.79
0.54
Day 3 Day 5 Day 7

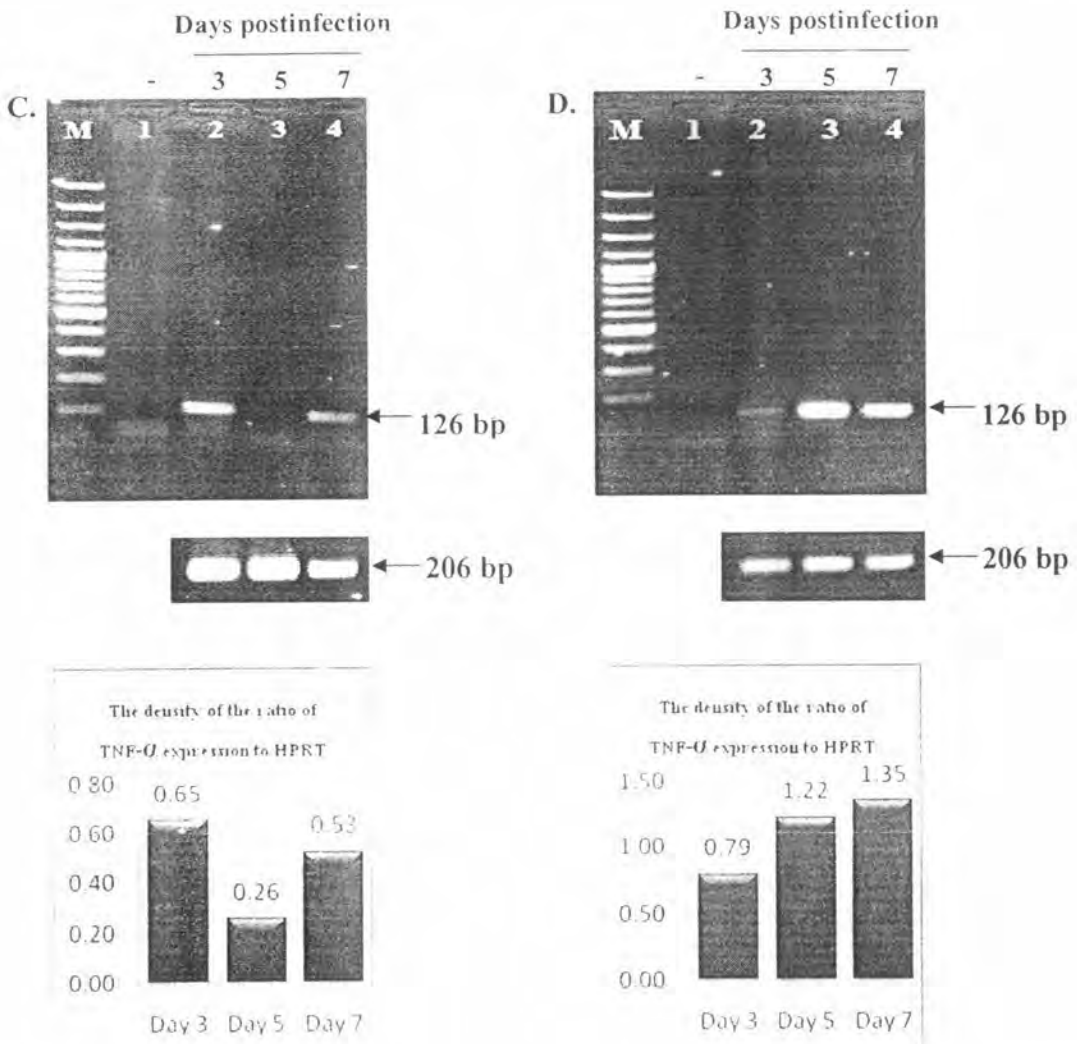


Figure 13 TNF-α mRNA expression in kidneys from hamsters infected with pathogenic *Leptospira*. Hamsters infected with pathogenic *Leptospira* were sacrificed at day 3, 5 and 7 post infection. RNA was extracted from kidney tissues and RT-PCR was performed for TNF-α mRNA expression. Lane M, 100-bp marker; Lane 1, negative control; Lanes 2, 3, and 4, kidneys from hamsters infected with pathogenic *Leptospira* at 3, 5, 7 days post infection, respectively. The lower figure showed HPRT mRNA expression in each sample and the graph underneath each figure demonstrates density ratio between bands from TNF-α and HPRT PCR products.

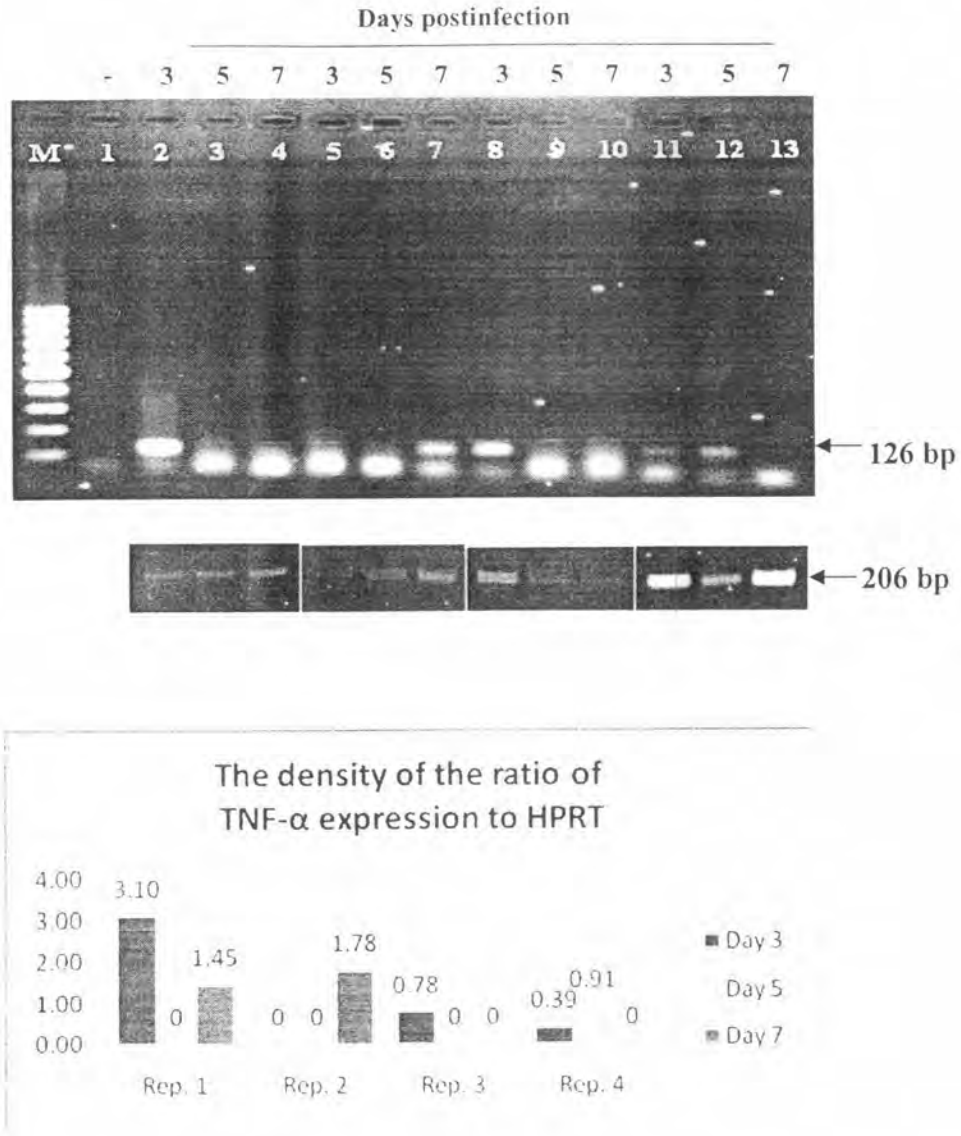


Figure 14 TNF-α mRNA expression in livers from hamsters infected with pathogenic *Leptospira*. Hamsters infected with pathogenic *Leptospira* were sacrificed at day 3 (Lanes 2, 5, 8, 11), 5 (Lanes 3, 6, 9, 12) and 7 (Lanes 4, 7, 10, 13) post infection. RNA was extracted from liver tissues and RT-PCR was performed for TNF-α mRNA expression. Lane M, 100-bp marker; Lane 1, negative control. The lower figure showed HPRT mRNA expression in each sample and the graph underneath each figure demonstrates density ratio between bands from TNF-α and HPRT PCR products.

4.3.3 TGF-β mRNA expression

The 186-bp PCR product demonstrated the presence of TGF-β mRNA expression. TGF-β mRNA expression was observed in kidneys from uninfected hamsters whereas the expression in liver tissues was undetectable (Figures 15A and Figure 15B, respectively). TGF-β mRNA expression was detected in both kidneys and livers from hamsters infected with pathogenic *Leptospira* (Figures 16-17).

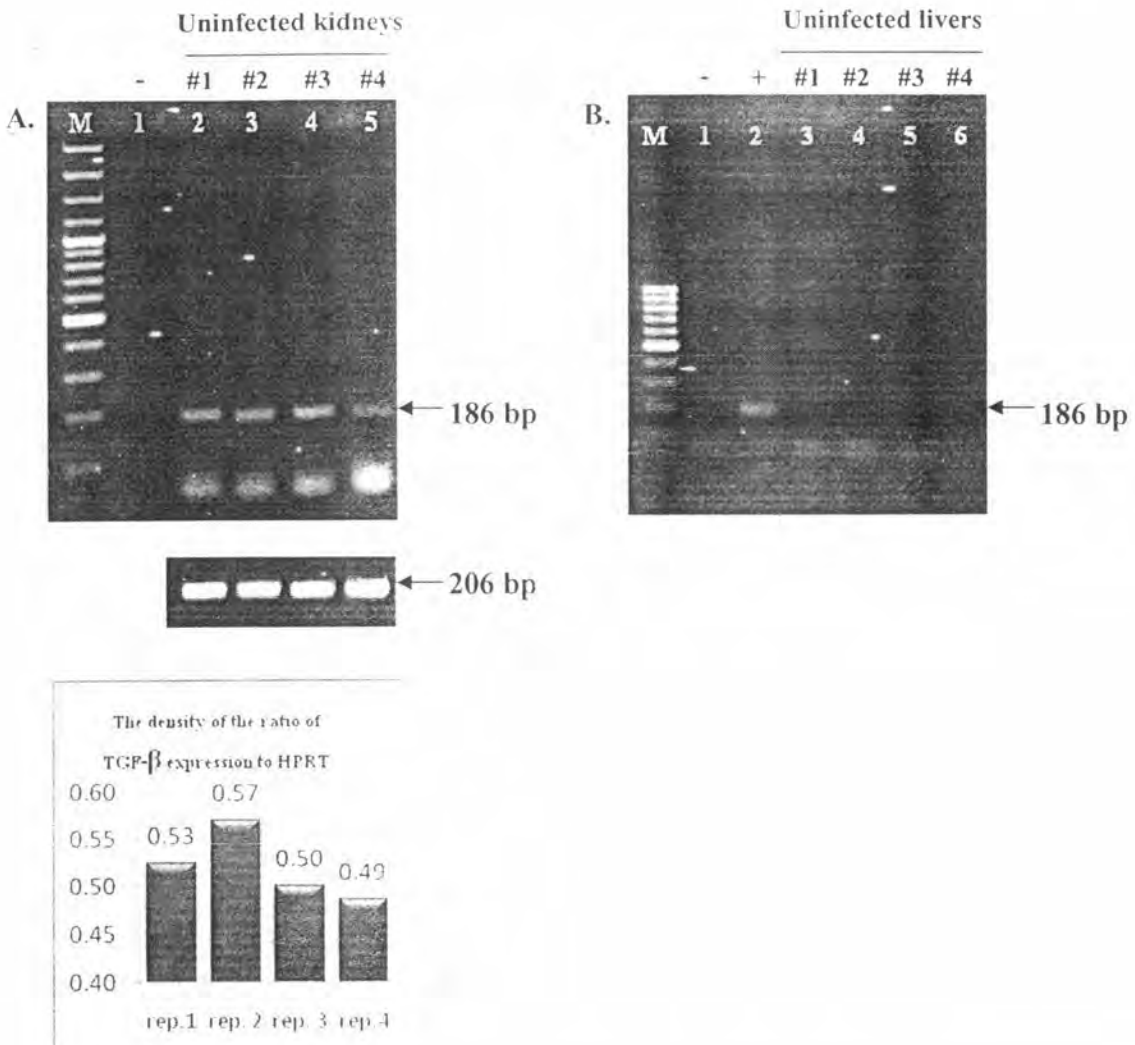
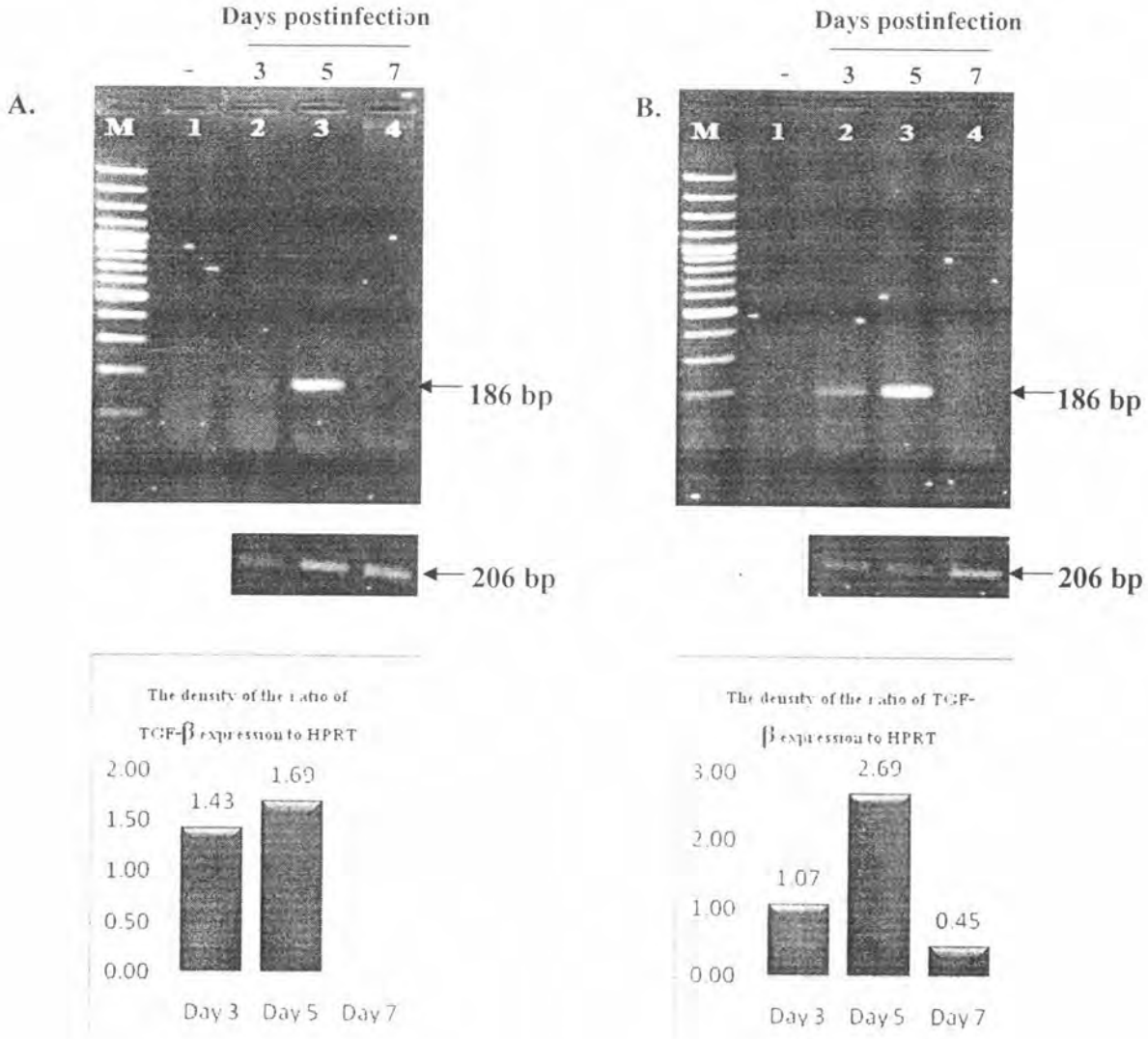


Figure 15 TGF-β mRNA expression in kidneys and livers of uninfected hamsters. cDNA was transcribed from RNA extracted from uninfected kidneys (15A) and livers (15B) of hamsters and PCR using TGF-β primers was performed as described in Materials and Methods. In Figure 15A, Lane M, 100-bp marker; Lane 1, negative control; Lanes 2, 3, 4, and 5, kidneys from four uninfected hamsters. In Figure 15B, Lane M, 100-bp marker; Lane 1, negative control; Lane 2, positive control; Lanes 3, 4, 5, and 6, livers from four uninfected hamsters. The lower figure showed HPRT mRNA expression in each sample and the graph underneath Figure 15A, demonstrates density ratio between bands from TGF-β and HPRT PCR products.


A.
Days postinfection
- 3 5 7
M 1 2 3 4
186 bp
206 bp
The density of the ratio of TGF-β expression to HPRT
2.00
1.50
1.00
0.50
0.00
1.43
1.69
Day 3 Day 5 Day 7
B.
Days postinfection
- 3 5 7
M 1 2 3 4
186 bp
206 bp
The density of the ratio of TGF-β expression to HPRT
3.00
2.00
1.00
0.00
1.07
2.69
0.45
Day 3 Day 5 Day 7

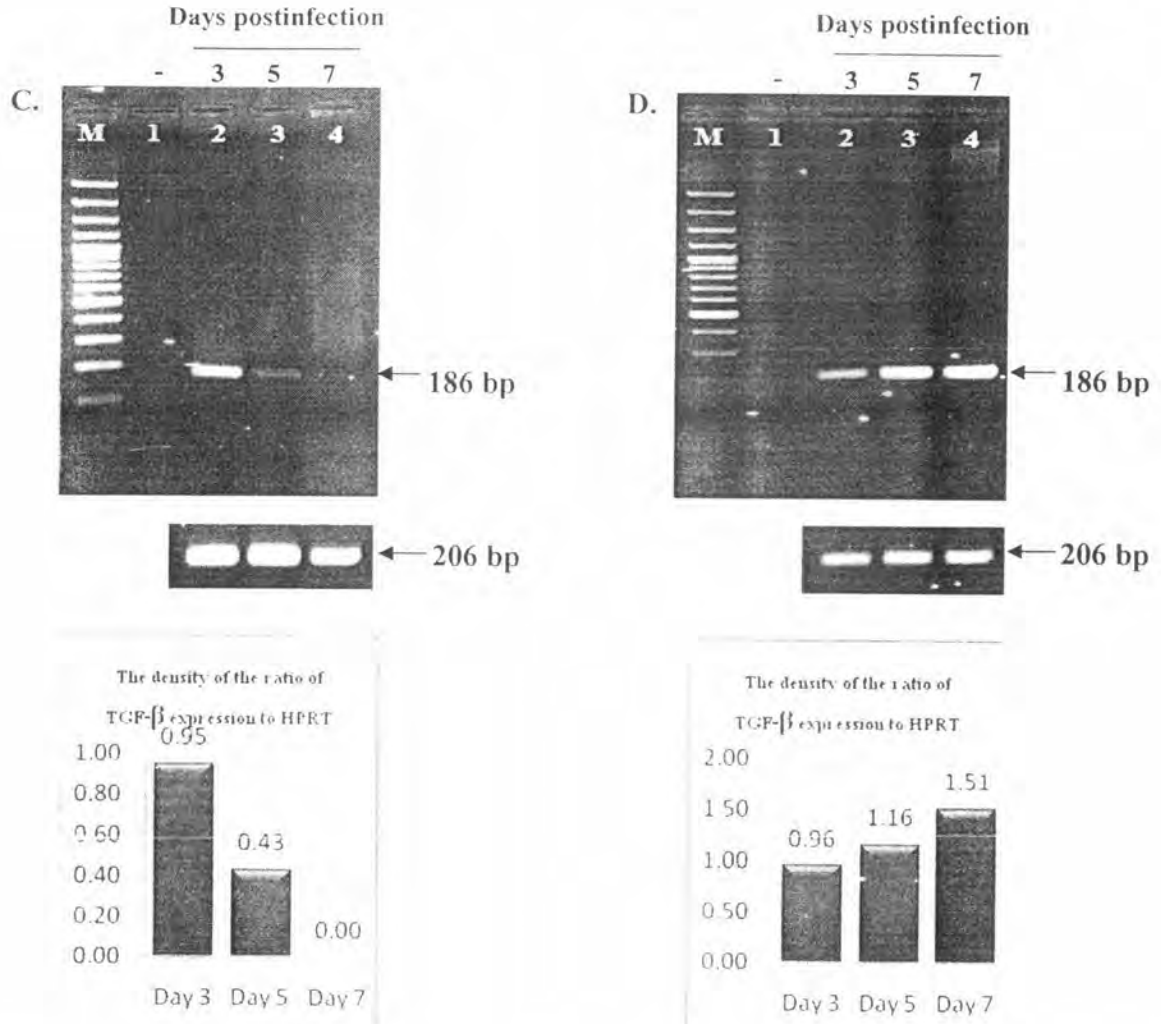
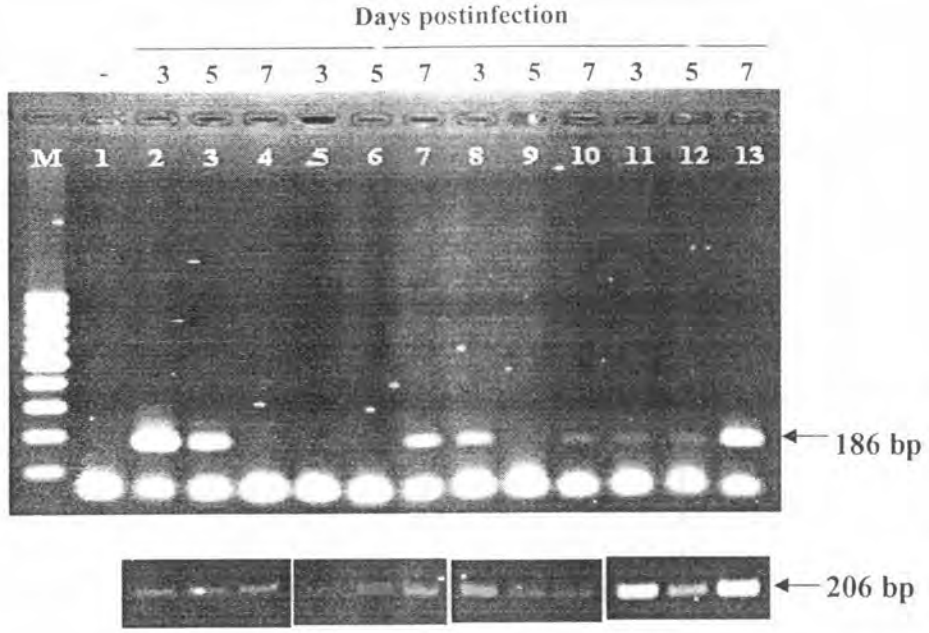


Figure 16 TGF-β mRNA expression in kidneys from hamsters infected with pathogenic *Leptospira*. Hamsters infected with pathogenic *Leptospira* were sacrificed at day 3, 5 and 7 post infection. RNA was extracted from kidney tissues and RT-PCR was performed for TGF-β mRNA expression. Lane M, 100-bp marker; Lane 1, negative control; Lanes 2, 3, and 4, kidneys from hamsters infected with pathogenic *Leptospira* at 3, 5, 7 days post infection, respectively. The lower figure showed HPRT mRNA expression in each sample and the graph underneath each figure demonstrates density ratio between bands from TGF-β and HPRT PCR products.



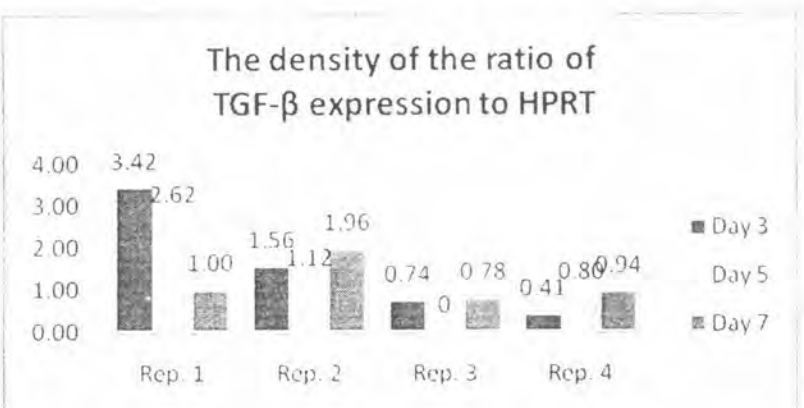


Figure 17 TGF-β mRNA expression in livers from hamsters infected with pathogenic *Leptospira*. Hamsters infected with pathogenic *Leptospira* were sacrificed at day 3 (Lanes 2, 5, 8, 11), 5 (Lanes 3, 6, 9, 12) and 7 (Lanes 4, 7, 10, 13) post infection. RNA was extracted from liver tissues and RT-PCR was performed for TGF-β mRNA expression. Lane M, 100-bp marker; Lane 1, negative control. The lower figure showed HPRT mRNA expression in each sample and the graph underneath each figure demonstrates density ratio between bands from TGF-β and HPRT PCR products.

4.3.4 IL-10 mRNA expression

The 163-bp PCR product indicated the presence of IL-10 mRNA expression. IL-10 gene mRNA expression was undetectable kidneys and livers of uninfected hamsters (Figure 18). In infected kidneys, the IL-10 mRNA expression was detected since day 3 post infection (Figure 19). However, IL-10 expression in infected livers was undetectable (Figure 20).

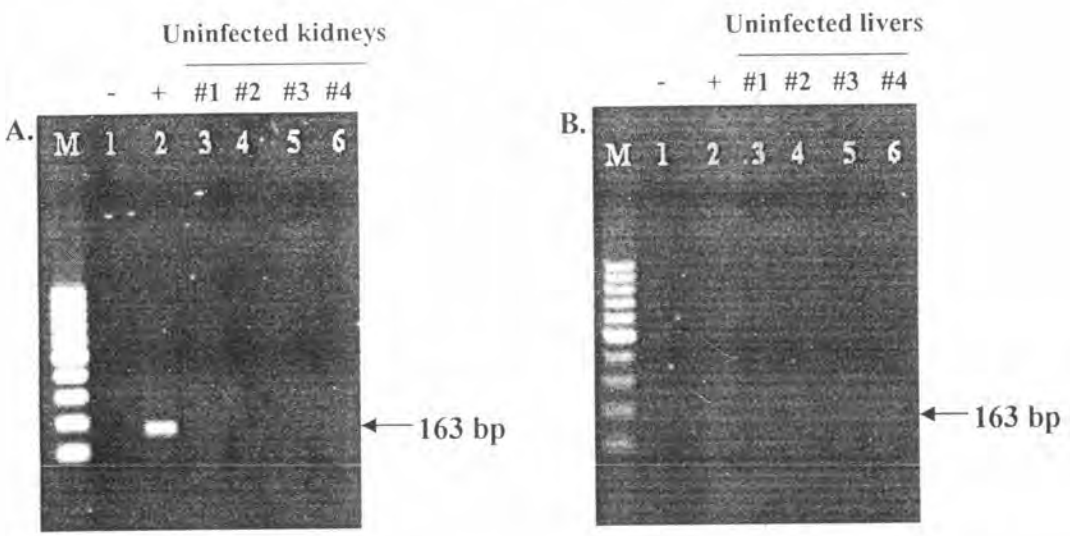
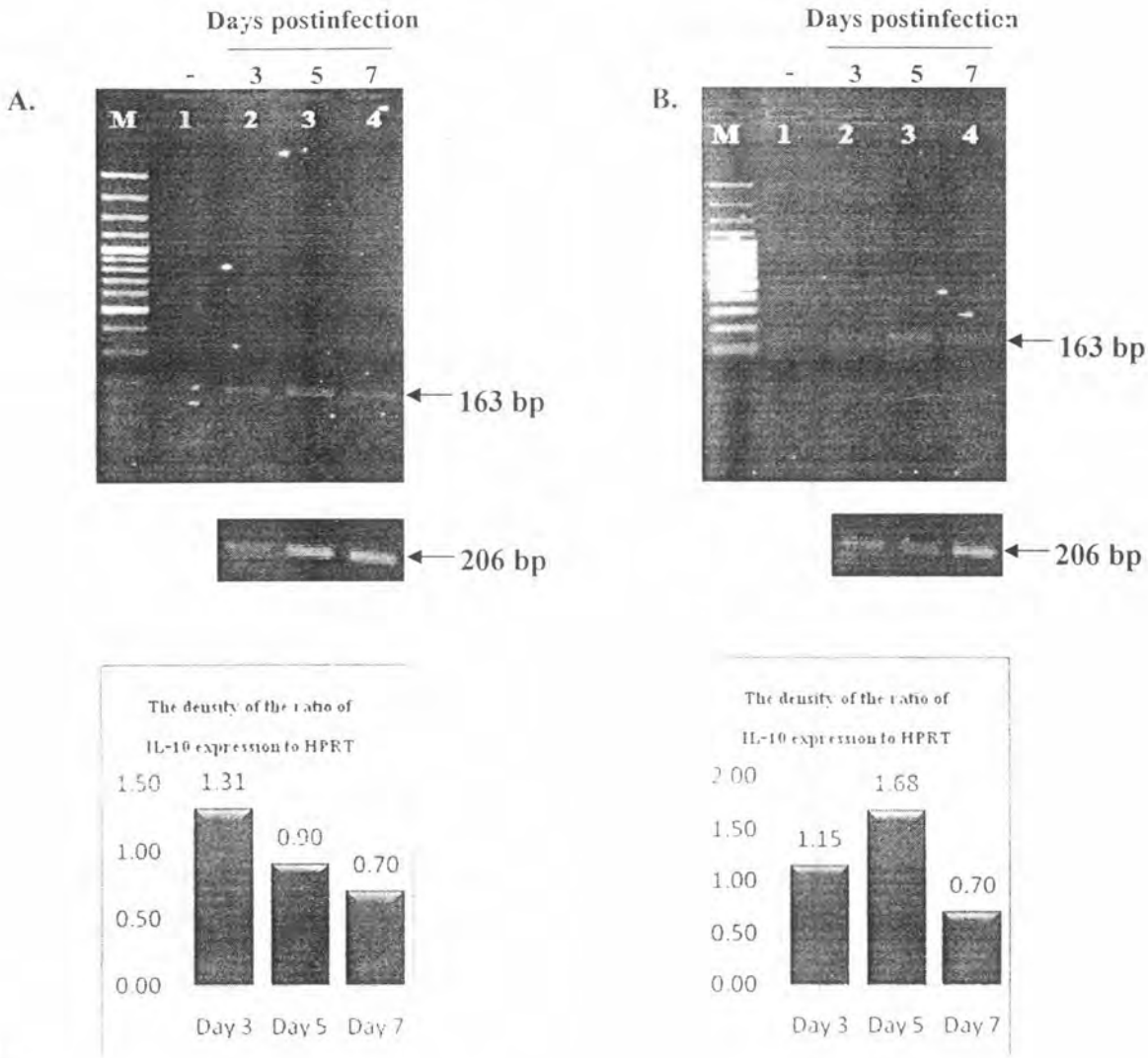


Figure 18 IL-10 expression in kidneys and livers of uninfected hamsters. cDNA was transcribed from RNA extracted from kidneys (18A) and livers (18B) of uninfected hamsters and PCR using IL-10 primers was performed as described in Materials and Methods. Lane M, 100-bp marker; Lane 1, negative control; Lane 2, positive control; Lanes 3, 4, 5, and 6, kidneys and livers from four uninfected hamsters.


Days postinfection
A.
- 3 5 7
M 1 2 3 4
163 bp
206 bp
Days postinfection
B.
- 3 5 7
M 1 2 3 4
163 bp
206 bp
The density of the ratio of
IL-10 expression to HPRT
1.50
1.00
0.50
0.00
1.31
0.90
0.70
Day 3 Day 5 Day 7
The density of the ratio of
IL-10 expression to HPRT
2.00
1.50
1.00
0.50
0.00
1.15
1.68
0.70
Day 3 Day 5 Day 7

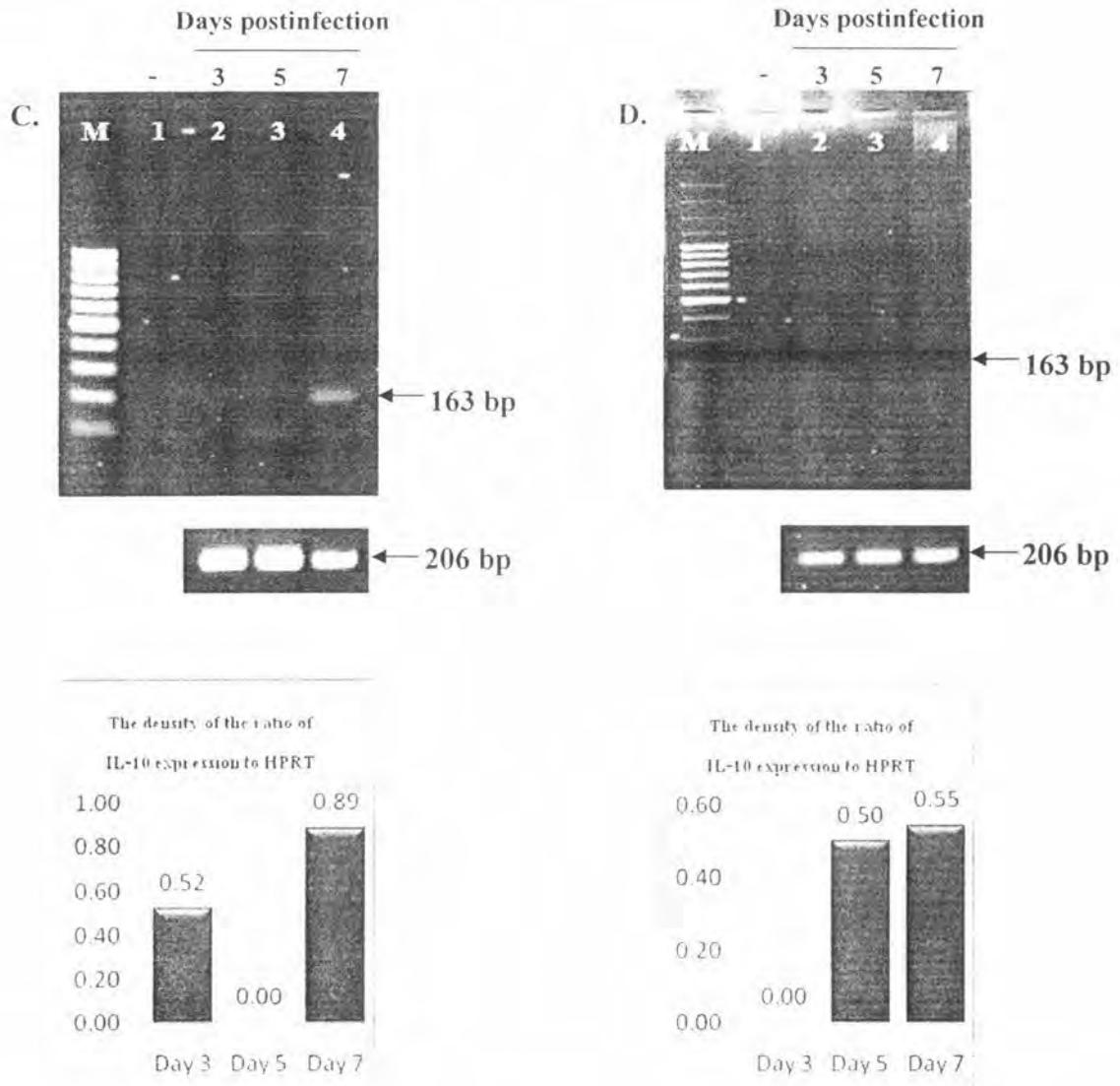


Figure 19 IL-10 mRNA expression in kidneys from hamsters infected with pathogenic *Leptospira*. Hamsters infected with pathogenic *Leptospira* were sacrificed at day 3, 5 and 7 post infection. RNA was extracted from kidney tissues and RT-PCR was performed for IL-10 mRNA expression. Lane M, 100-bp marker; Lane 1, negative control; Lanes 2, 3, and 4, kidneys from hamsters infected with pathogenic *Leptospira* at 3, 5, 7 days post infection, respectively. The lower figure showed HPRT mRNA expression in each sample and the graph underneath each figure demonstrates density ratio between bands from IL-10 and HPRT PCR products

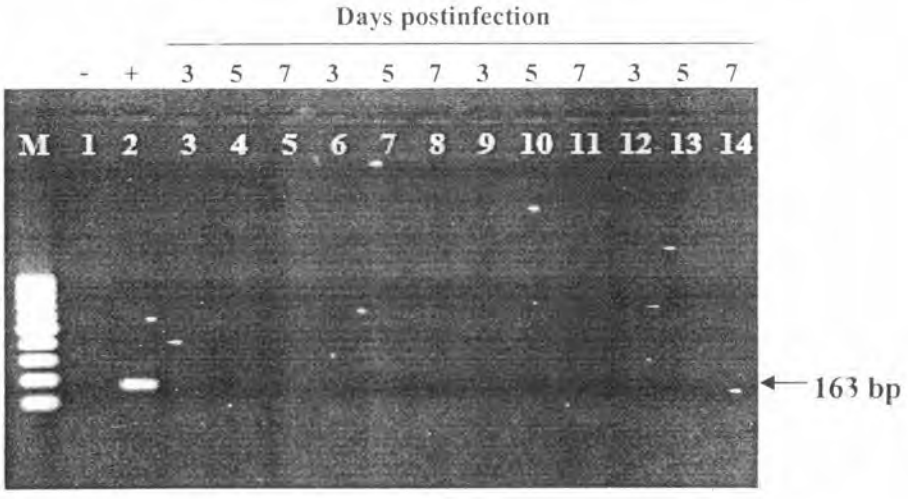


Figure 20 IL-10 mRNA expression in livers from hamsters infected with pathogenic *Leptospira*. Hamsters infected with pathogenic *Leptospira* were sacrificed at day 3 (Lanes 3, 6, 9, 12), 5 (Lanes 4, 7, 10, 13) and 7 (Lanes 5, 8, 11, 14) post infection. RNA was extracted from liver tissues and RT-PCR was performed for IL-10 mRNA expression. Lane M, 100-bp marker; Lane 1, negative control; Lane 2, positive control. The graph underneath each figure demonstrates density ratio between bands from IL-10 and HPRT PCR products.

4.3.5 IP-10 mRNA expression

The expression of IP-10 mRNA was demonstrated by the presence of 509-bp PCR product. IP-10 expression was detected in kidneys (Figure 21A) from uninfected hamsters whereas the expression was undetectable in liver tissues (Figure 21B). In infected kidneys, the IP-10 mRNA expression was detected since day 3 post infection (Figure 22). The relative level of IP-10 mRNA expression was the highest when compared with the expression of other cytokines in infected kidneys. However, in infected livers, IP-10 mRNA expression was undetectable (Figure 23).

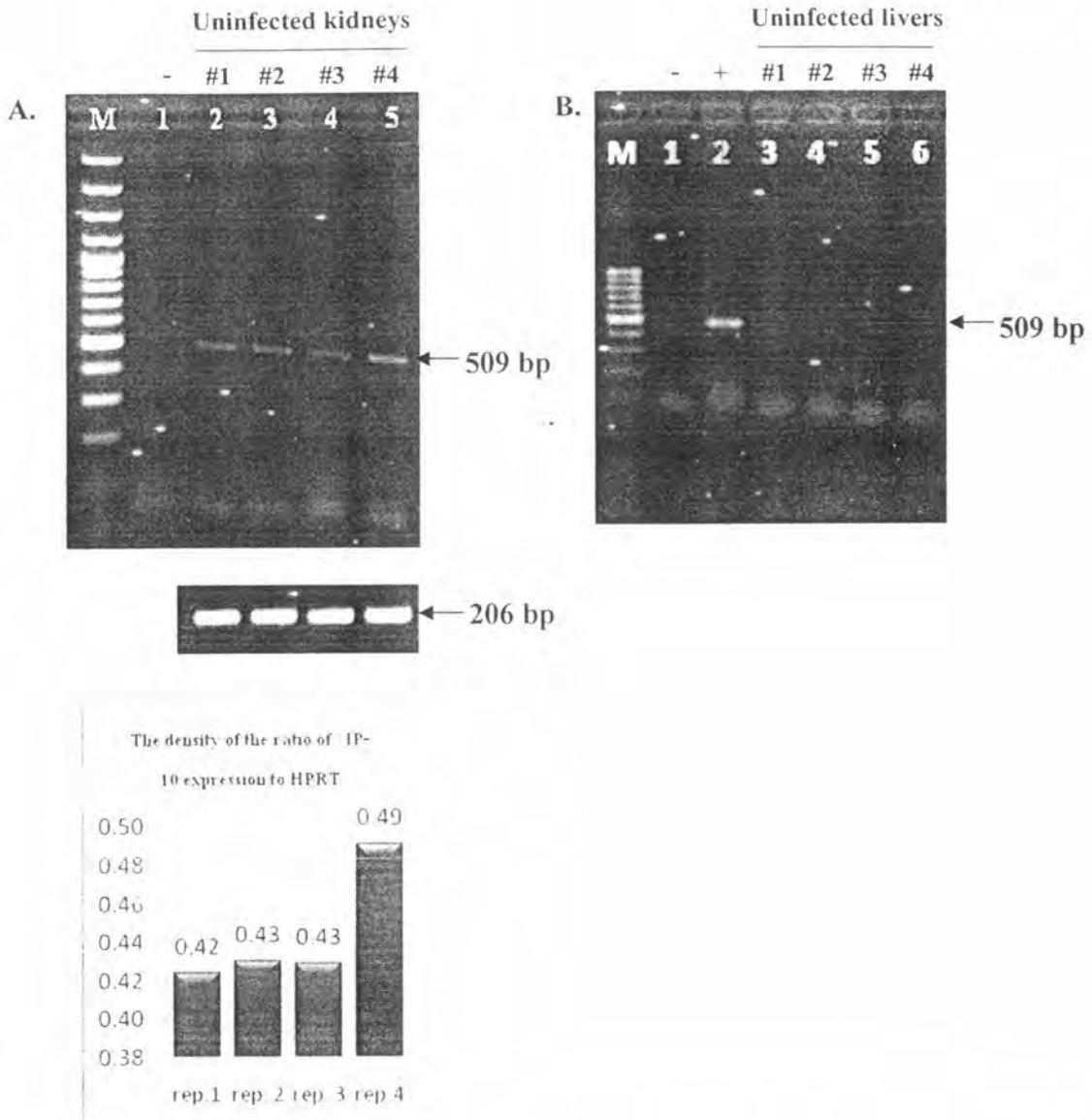
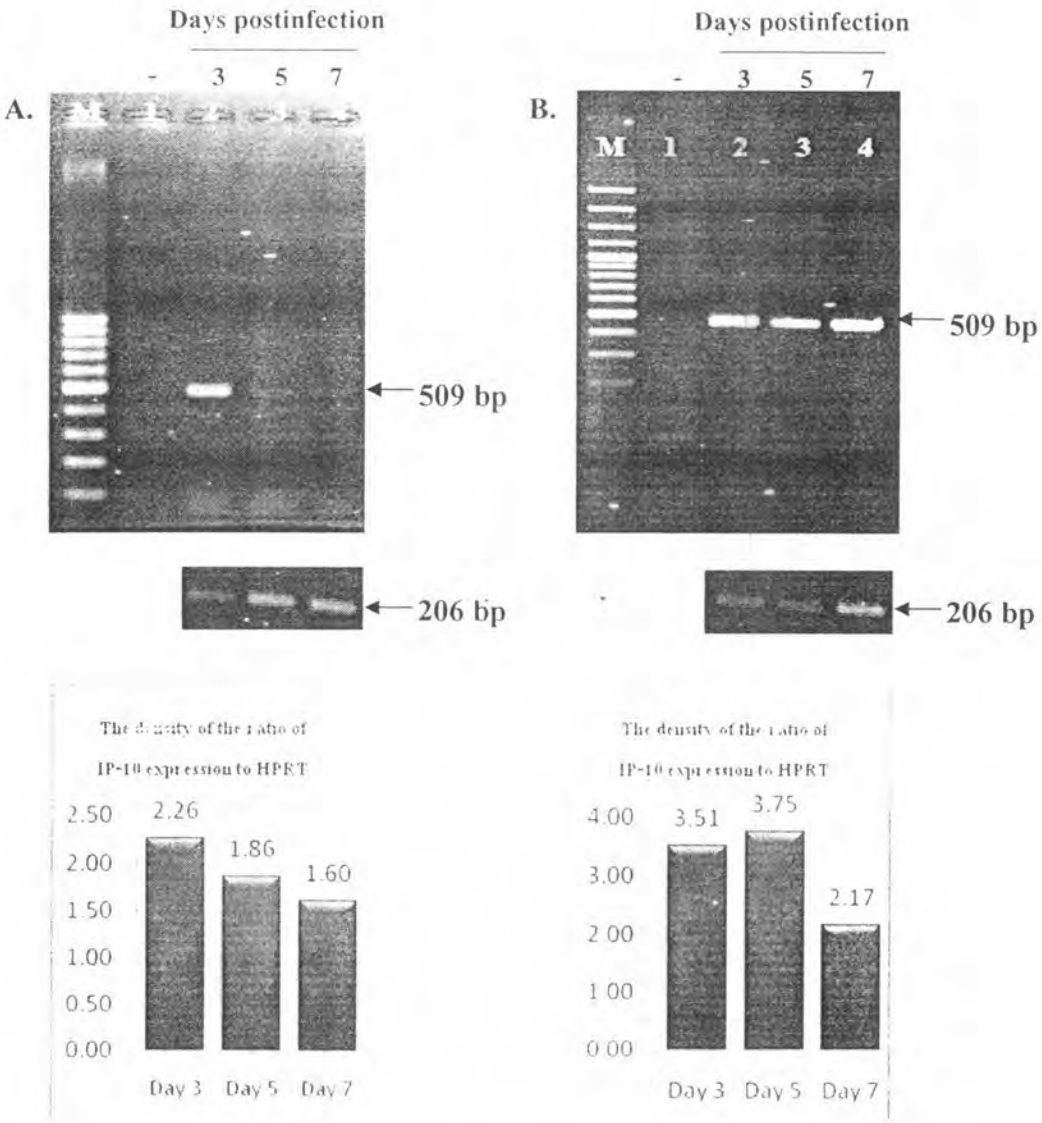


Figure 21 IP-10 mRNA expression in kidneys and livers from uninfected hamsters. cDNA was transcribed from RNA extracted from kidneys (Figure 21A) and livers (Figure 21B) of uninfected hamsters and PCR using IP-10 primers was performed as described in Materials and Methods. In Figure 21A, Lane M, 100-bp marker; Lane 1, negative control; Lanes 2, 3, 4, and 5, kidneys from four uninfected hamsters. In Figure 21B, Lane M, 100-bp marker; Lane 1, negative control; Lane 2, positive control; Lanes 3, 4, 5, and 6, livers from uninfected hamsters. The lower figure showed HPRT mRNA expression in each sample and the graph underneath Figure 21A demonstrates density ratio between bands from IL-10 and HPRT PCR products.


Days postinfection
- 3 5 7
A.
509 bp
206 bp
Days postinfection
- 3 5 7
B.
M 1 2 3 4
509 bp
206 bp
The density of the ratio of
IP-10 expression to HPRT
2.50
2.00
1.50
1.00
0.50
0.00
2.26
1.86
1.60
Day 3 Day 5 Day 7
The density of the ratio of
IP-10 expression to HPRT
4.00
3.00
2.00
1.00
0.00
3.51
3.75
2.17
Day 3 Day 5 Day 7

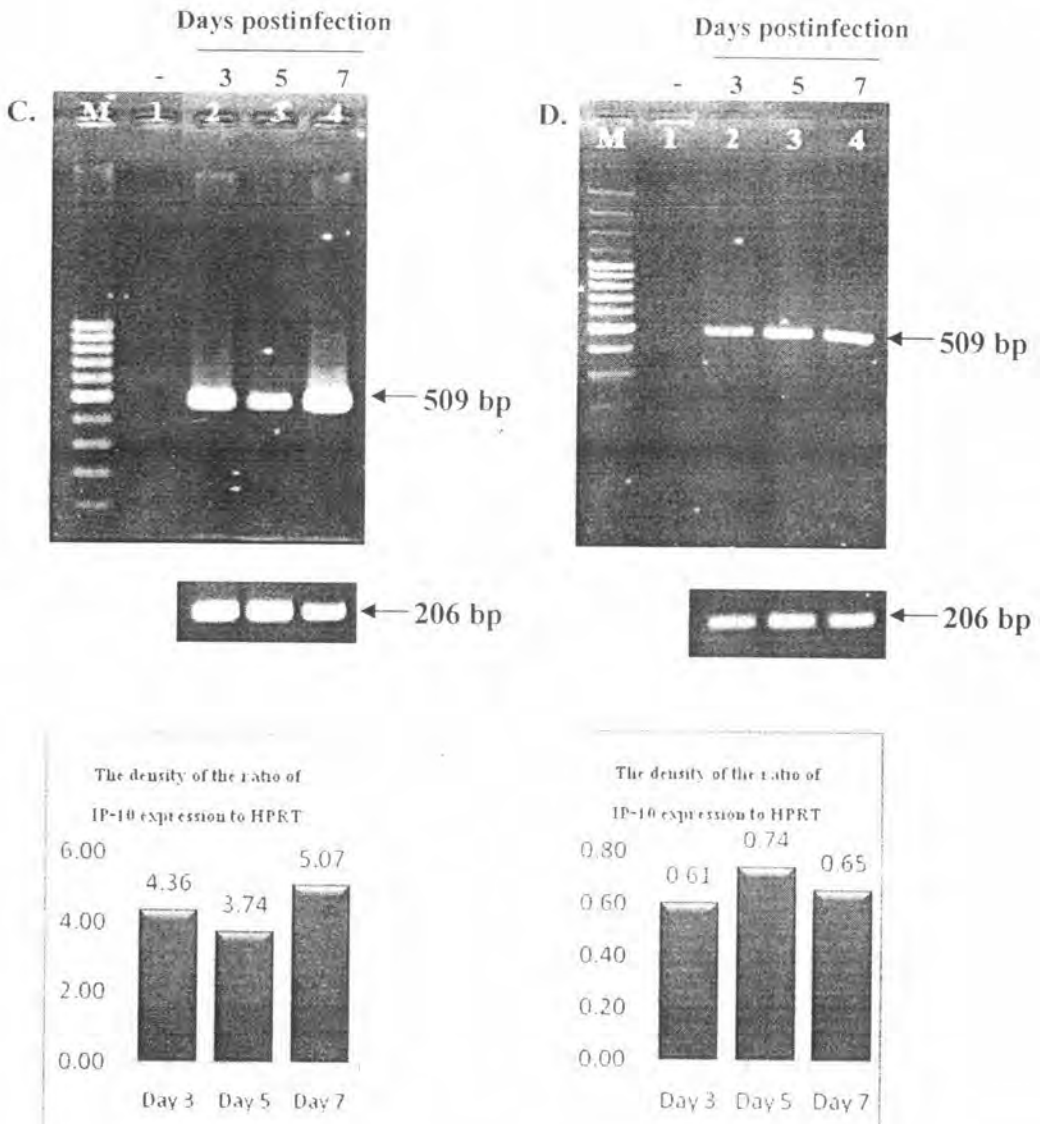


Figure 22 IP-10 mRNA expression in kidneys from hamsters infected with pathogenic *Leptospira*. Hamsters infected with pathogenic *Leptospira* were sacrificed at day 3, 5 and 7 post infection. RNA was extracted from kidney tissues and RT-PCR was performed for IP-10 mRNA expression. Lane M, 100-bp marker; Lane 1, negative control; Lanes 2, 3, and 4, kidneys from hamsters infected with pathogenic *Leptospira* at 3, 5, 7 days post infection, respectively. The lower figure showed HPRT mRNA expression in each sample and the graph underneath each figure demonstrates density ratio between bands from IP-10 and HPRT PCR products.

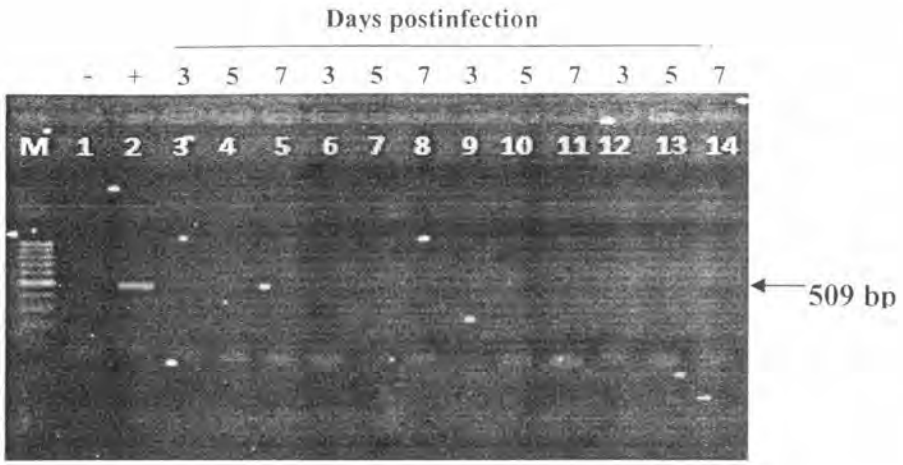


Figure 23 IP-10 mRNA expression in livers of hamsters infected with pathogenic *Leptospira*. Hamsters infected with pathogenic *Leptospira* were sacrificed at day 3 (Lanes 3, 6, 9, 12), 5 (Lanes 4, 7, 10, 13) and 7 (Lanes 5, 8, 11, 14) post infection. RNA was extracted from liver tissues and RT-PCR was performed for IL-10 mRNA expression. Lane M, 100-bp marker; Lane 1, negative control; Lane 2, positive control. The graph underneath each figure demonstrates density ratio between bands from IP-10 and HPRT PCR products.

4.4 Relative expression levels of LipL32 in kidneys from hamsters infected with pathogenic *Leptospira* determined by real-time PCR

As demonstrated earlier that LipL32 mRNA expression could be observed in kidneys and livers of infected hamsters, the level of expression was further determined using realtime PCR as described in Materials and Methods. Since the kidney is an organ commonly affected in Leptospirosis, the level of LipL32 expression was further studied in kidney tissues. The relative expression level of LipL32 normalized with 16S rRNA was calculated and average ratio of LipL32 expression in infected over uninfected group was 7.96, 7.43, and 7.92 on days 3, 5 and 7, respectively (Figure 24). LipL32 expression in infected/uninfected group was 8.81, 6.74, 7.48 and 8.83 at day 3, 9.8, 6.95, 4.81 and

8.18 at day 5 and 10.37, 7.93, 6.87 and 6.54 at day 7 post-infection.

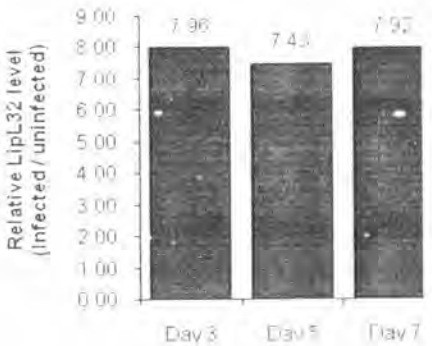


Figure 24 The relative expression level of LipL32 to 16S rRNA in kidneys of hamsters infected with pathogenic *Leptospira* by real-time quantitative SYBR-PCR. cDNA was transcribed from RNA extracted from kidneys and SYBR Green real-time PCR was performed to demonstrate the relative expression level of LipL32 to 16SrRNA. The graph demonstrated the average folds of LipL32 expression in kidneys of infected hamsters on 3, 5 and 7 days post-infection.

4.5 Relative expression levels of IP-10 in kidneys from hamsters infected with pathogenic *Leptospira* determined by real-time PCR

As shown by RT-PCR, IP-10 mRNA expression was observed in both uninfected and infected kidneys whereas the expression in liver tissues was undetectable. Since the expression of IP-10 seemed to be the strongest among other cytokines detected in kidneys, real-time PCR was performed to quantitate mRNA expression of this chemokine in kidneys. The relative expression level of IP-10 normalized with HPRT was calculated and average ratio of IP-10 expression in infected over uninfected group was 3.91, 7.69 and 12.45 on days 3, 5 and 7, respectively (Figure 25). IP-10 expression in infected/uninfected group was 3.50, 2.73, 2.54 and 6.86 folds on day 3, 0.97, 11.37, 0.92 and 17.48 folds on day 5, and 8.92, 6.62, 20.93 and 13.34 folds on day 7 postinfection.

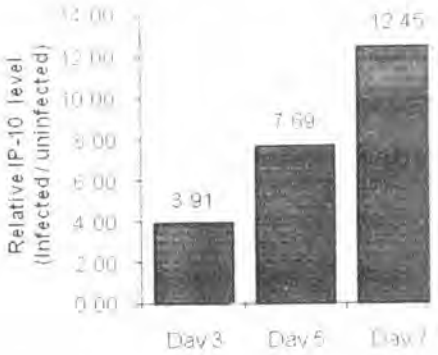


Figure 25 The relative expression level of IP-10 to HPRT in kidneys of hamsters infected with pathogenic *Leptospira* by real-time quantitative SYBR-PCR. cDNA was transcribed from RNA extracted from kidneys and SYBR Green real-time PCR was performed to demonstrate the relative expression level of IP-10 to HPRT. The graph demonstrated the average folds of IP-10 expression relative to HPRT in kidneys of infected hamsters on 3, 5 and 7 days post-infection over uninfected kidney tissues.