Original article

LipL21 mRNA expression in lungs of hamsters infected with pathogenic *Leptospira*

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Abstract

Objective: Pulmonary haemorrhage is an increasing cause of death in leptospirosis patients. However, molecular mechanism underlying pathologies in this organ is not clearly understood. It has been shown that sodium transport was disturbed following *Leptospira* infection. LipL21 is the second abundant outer membrane protein found only in pathogenic *Leptospira*. Its expression in *vivo* has been shown which suggests that this protein may be involved in survival in hosts or pathogenesis. However, the expression of this protein in host organs and its role in lung pathology has not been demonstrated. In this study we demonstrated the expression of LipL21 in lungs of hamsters infected with pathogenic *Leptospira*. **Methods**: Lung tissues were collected from Golden Syrian hamsters injected with *Leptospira interrogans* serovar Pyrogenes at days 3, 5 and 7 post-infection. Four hamsters were used for each time point. Lungs from non-infected hamsters were collected as a control group. LipL21 mRNA expression in lung tissues was investigated by reverse transcription and nested PCR. **Results**: LipL21 mRNA expression was detected in all lung tissues from hamsters infected with pathogenic *Leptospira*. No PCR product was detected when tissues from non-infected hamsters were investigated. **Conclusion**: Our data demonstrated that LipL21 is expressed in lungs of hamsters infected with pathogenic *Leptospira*. Additional experiments such as quantitation and localization of LipL21 expression in lungs will provide further information whether this protein is involved in pathogenesis.

Keywords: Leptospirosis; *Leptospira*; LipL21; Lung haemorrhage

INTRODUCTION

Leptospirosis, an infectious disease caused by spirochetal bacteria named *Leptospira*, is a zoonosis found worldwide. The disease is usually reported in tropical to subtropical area, however, *Leptospira* also initiates an illness in western countries[1]. *Leptospira* normally live in a urinary tract of wide-ranged animals, mainly rodent and swine. Humans acquire

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leptospirosis from exposure to contaminated urine or water. Disease manifestations of leptospirosis patients vary from mild flu-like symptoms to severe illness involving several organs [2]. A febrile illness might occur from bacteraemia in the first week of an infection then a specific antibody against the bacteria would rise up in order to excrete them through urine. However, *Leptospira* can still remain in a body, localize at other organs, disturb host functions and are causes of presented symptoms. The most eminent symptom in leptospirosis is called "Weil's disease" which is an effect of the bacteria to liver and kidney[3]. It is characterized by jaundice, renal failure and haemorrhage. Pulmonary bleeding is a poor prognosis of the disease, the patient showed dyspnoea and haemoptysis. These conditions were unrelated to jaundice. For haemorrhage describe, thrombocytopenia [4,5] which is remarked in most patients seem to be the best answers, however, disseminated intravascular coagulation is not the process of pathogenesis according to the study of Edwards CN and his colleague [6]. Autoantibodies or chemokines induced by bacterial virulence were also proposed to be the causes of pathologies [7,8].

Leptospirosis has been increasingly reported as a cause of severe pulmonary haemorrhage syndrome and fatality rate is over 50 % [9-11]. The reason for this increasing and mechanisms of pathologies are still unknown. Several *Leptospira* components and their roles in pathogenesis have been studied. Outer membrane proteins are the most widely studied because it is likely that they are involved in host attachment and are accessible to host immune recognition. The expression of these proteins *in vivo* implies that they are at least involved in survival in susceptible hosts or even are virulence factors. These in vivo expressed components should be an ideal target for bactericidal and vaccine development.

LipL32 is the most abundant leptospiral outer membrane protein (OMP) and is the most widely studied component. However, it has been recently shown that mutation of this protein has no effect on acute or chronic infection [12]. LipL21, a 21 kDa lipoprotein, is the second abundant outer protein identified in *Leptospira*. It is conserved among the pathogenic serovars. LipL21 reacted with sera from infected hamsters which suggested that this protein is expressed *in vivo* and induced antibody response. The studies of LipL21 detection for leptospirosis diagnostic tool development has been reported [13-15]. Moreover, DNA vaccine containing full LipL21 gene segment demonstrated protection in guinea pigs challenged intraperitoneally with *L. interogans* sorevar Lai which suggested that this protein is a good candidate for further vaccine development [16].

However, there is not report on the involvement of LipL21 in pathogenesis especially in lungs. In this study, we investigated whether LipL21 gene was expressed in lungs of hamsters infected with pathogenic *Leptospira*.

MATERIALS AND METHODS

Hamster injection

The animal experiments were conducted under the approval of the Ethical Research Committee, Faculty of Medicine, Chulalongkorn University. Twelve Golden Syrian hamsters were injected intraperitoneally with 10^8 *Leptospira interrogan*serovar Pyrogenes and then were scarified 3, 5 and 7 days post-infection. Four hamsters were sacrificed for each timepoint. The tissues were collected and preserved in RNA later solution for RNA extraction and in formalin for pathological examination. Four uninfected hamsters were used as a control group.

PCR for LipL21 detection

DNA was extracted from 23 reference pathogenic *Leptospira interrogans* serovars and a non-pathogenic *Leptospira biflexa* serovar patoc I using DNaZol (Invitrogen) according to the manufacturer instruction. PCR was performed with LipL21-F and Lip21R primers using the thermal cycling protocol as follow: 94 ℃ for 1 min, 55 ℃ for 1 min and 72 ℃ for 1min for 30cycles. PCR products were visualized on1.5% agarose gel staining with ethidium bromide. The detection of 341-bp PCR product indicated the presence of LipL21. DNA extracted from *Leptospira biflexa* serovar Patoc I was also tested in this experiment.

Reverse transcription

Total RNA were extracted from 0.02 g of lung tissue using TRIZOL reagent (Invitrogen, CA) according to the manufacturer instruction. One microgram of RNA was treated with RNase-free DNase I (Fermentas, MD) for residual DNA removal before using in cDNA synthesis. cDNA synthesis was performed with random hexamer and M-MuLV reverse transcriptase (Fermentas) at 42°C for 60 min.

RT-PCR for detection of 16S rRNA expression

Complementary DNA was amplified using primers specific to 16S rRNA with the thermal cycling protocol as follow: 94°C for 1 min, 55°C for 1 min and 72°C for 1 min for 30 cycles; for LipL21 amplification PCR products were visualized by 1.5 % agarose gel electrophoresis. The presence of 290-bp indicated the presence of 16S rRNA expression.

Nested RT-PCR for detection of LipL21 expression

Nested PCR for detection of LipL21 expression in lung tissues was perform using the same cDNA and thermal cycling protocol as used for 16S rRNA detection. LipL21-F and LipL21-R primers were used for the first round PCR and LipL21-F and LipL21-RR were used for the second round. The 341- and 225-bp PCR products were the expected sizes of the first and second round PCR, respectively. Sequences of all primers used in this study and expected PCR product sizes were shown in Table 1.

RESULTS

Detection of LipL21 in pathogenic *Leptospira*

PCR for LipL21 detection was performed to confirm that LipL21 could be detected in all pathogenic *Leptospira*. DNA from 23 reference pathogenic serovars routinely used in laboratory diagnosis in Thailand and a non-pathogenic serovar were tested for LipL21 detection. As shown in Figure 1A and 1B, the 341-bp product was detected in DNA from all 23 pathogenic serovars but not in DNA from non-pathogenic *Leptospira*.

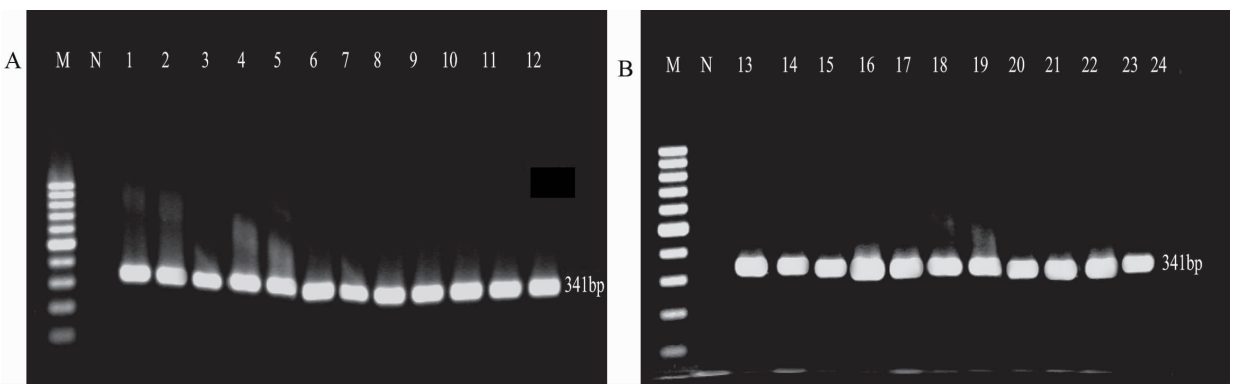

Figure 1 LipL21 detection in reference pathogenic *Leptospira*.
DNA extracted from 23 pathogenic references and a non-pathogenic serovar, was amplified by PCR for LipL21 detection. M, 100-bp marker; N, reagent control; Lanes 1-23 were DNA from *L. interrogans* serovar Bratislava, Autumnalis, Ballum, Battaviae, Canicola, Cellodoni, Cynopteri, Djasiman, Grippotyphosa, Hebdhomadis, Icterohaemorrhagiae, Javanica, Louisiana, Manhao, Mini, Panama, Pomona, Pyrogenes, Ranarum, Sarmin, Seijoe, Shermani and Tarassovi, respectively; Lane 24 was DNA from *L. biflexa* serovar Patoc I. The 341-bp band was detected in DNA from all reference pathogenic serovars (Lanes 1-23) whereas there was no band detected in DNA which was from non-pathogenic *Leptospira*.

16S rRNA expression in lungs of hamsters infected with pathogenic *Leptospira*

RT-PCR for 16S rRNA expression was performed to demonstrate the presence of *Leptospira* in lungs. 16S rRNA expression was detected in all lung tissues from infected hamsters (Figure 2) whereas no PCR product was observed in uninfected tissues (data not shown).

LipL21 gene expression in lung of hamsters infected with pathogenic *Leptospira*

For the detection of LipL21 gene expression, RT and

nested PCR were done because the expression could not be observed when regular PCR was performed. The expected sizes of PCR products of the first and second rounds PCR was 341 and 225 bp, respectively. Both bands were observed when the positive control which was cDNA from RNA extracted directly from *Leptospira*, was used. LipL21 gene expression was detected in all 12 samples from lung tissues of infected hamsters (Figure 3A-D). There was no LipL21 expression detected when lung tissues from uninfected hamsters were tested (data not shown).

Table 1 Sequences of primers used in this study and expected PCR product size.

Primers	Sequences	Product size (bp)
LipL21-F	5′-GCA GCT TGT TCC AGT ACT GAC-3′	341
LipL21-R	5′-CGA TTA CAG ATG CAG TAG CTT C-3′	341
LipL21-RR	5′-GTG GAT TGC ATC ATC GCT TGA C-3′	225
16S rRNA-F	5′-CAAGTCAAGCGGAGTAGCAA-3′	290 [22]
16S-rRNA R	5′-CTTAACCTGCTGCCTCCCGTA-3′	290 [22]

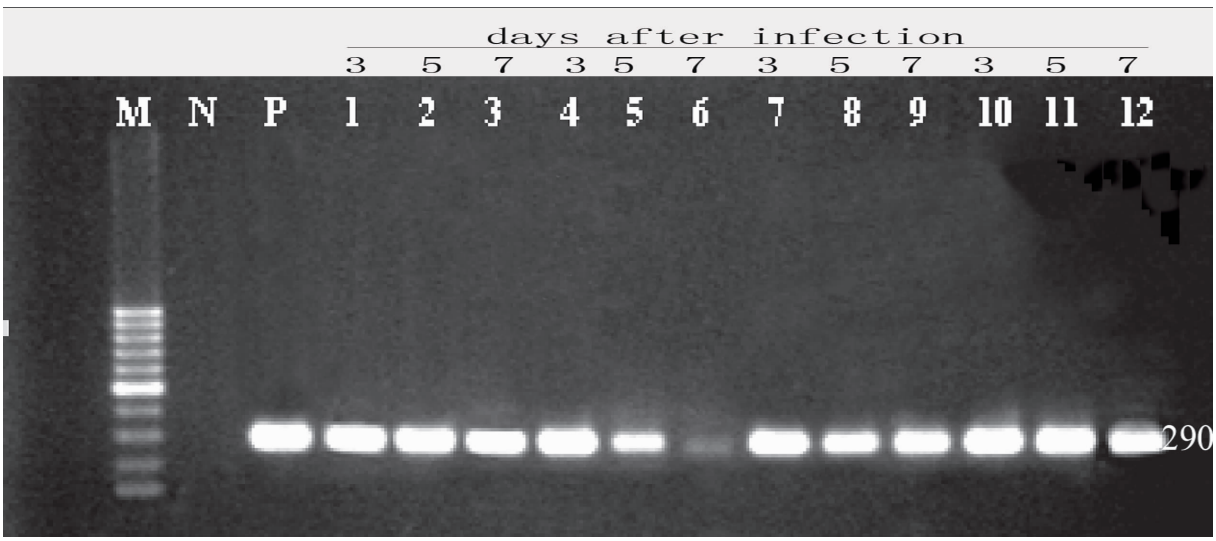

Figure 2 16S rRNA gene expression in hamsters′ lungs infected with pathogenic *Leptospira*.
The samples were investigated for the presence of *Leptospira* 16S rRNA by RT-PCR. M: 100 bp marker; N: reagent control; P: positive control; Lanes 1, 4, 7 and 10 are tissues at 3 days after infection; 2, 5, 8 and 11 are 5 days after infection and 3, 6, 9 and 12 are 7 days after infection. The arrow indicated the 290-bp PCR product of 16S rRNA.

DISCUSSION

Although leptospiosis and its etiologic agent have been discovered for quite some time, the mechanisms of pathogenesis are still poorly understood. The mechanism of pathologies in leptospirosis may involve direct effect of the spirochete components and host′s immune response to *Leptospira*. It is interesting why some leptospirosis patients have no or mild symptoms whereas some can develop severe illness involving multi-organ damages. The study of the genes that are expressed in target organs will provide further information on bacterial factors that may involve in pathogenesis in those organs.

Because lung haemorrhage is one of the manifestations found in severe cases of leptospirosis, we are

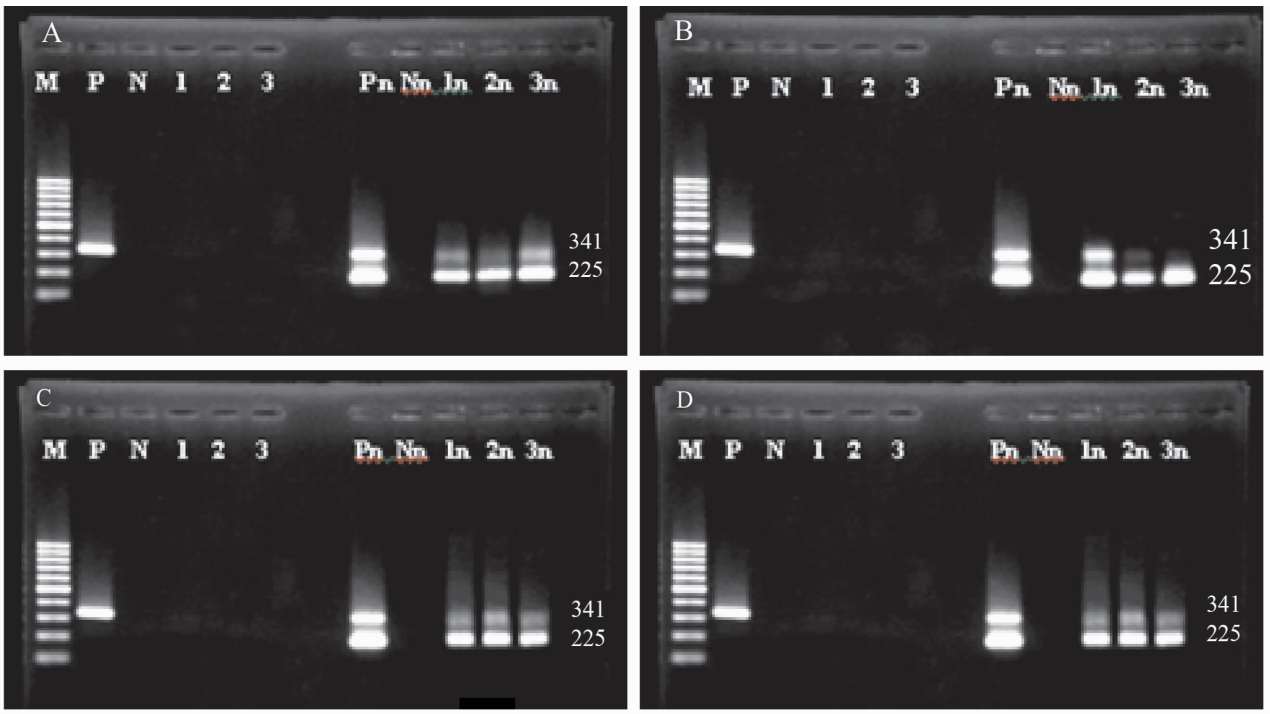

Figure 3 LipL21 gene expression in hamsters' lungs infected with pathogenic *Leptospira*.
LipL21 expression in hamsters' lungs at 3, 5 and 7 days after Leptospira infection in 4 replications was investigated by nested RT-PCR as described in Materials and Methods. M, 100-bp marker; P, Positive control; N, reagent control; Lane 1, lung tissue at day 3; Lane 2, lung tissue at day 5; Lane 3, lung tissue at day 7. Pn, Nn, 1n, 2n, 3n were results of the same samples after nested PCR. The upper and lower bands observed were 341- and 225-bp PCR products, respectively.

interested in investigating which *Leptospira* components are involved in lung pathology. It has been shown that *Leptospira* glycoprotein inhibited Na, K-ATPase activity [17,18]. *Leptospira* infection dysregulated sodium transport by decreasing expression of epithelium sodium channel and up-regulating Na-K-2Cl co-transporter expression in the lungs of infected hamsters which suggested that alteration of pulmonary function may increase lung injury by *Leptospira* infection [19,20].

Several other *Leptospira* components have been investigated; however, there was no report on the involvement of LipL21, the second abundant *Leptospira* outer protein, in lung pathogenesis. *Leptospira* interrogans serovar Pyrogenes, the isolate previously shown to induce pathologies in hamsters was used in this study[21,22]. Histopathological examination of lung tissues demonstrated pulmonary atelectasis which characterized by collapsing of alveolar spaces on lung tissues collected on day 3 post-infection. Pulmonary haemorrhage was observed on both tissues from day 5 and day 7; however, the degree of hemorrhage was higher in day-7 than in day-5 samples (data not shown). We established the nested PCR assay for detection of LipL21 mRNA expression. The assay was specific to infected tissues since there was no LipL21 expression detected in uninfected tissues. LipL21 mRNA expression was detected since day 3 after infection. Lung pathology was also observed in samples from day 3 post-infection and the severity seemed to increase on day 5 and day 7. However, quantitation and localization of LipL21 expression in lung tissues are needed to be done to further investigate the involvement of LipL21 expression in lung pathology.

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