In vivo gene expression and immunoreactivity of Leptospira collagenase

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ABSTRACT

Pulmonary hemorrhage is an increasing cause of death of leptospirosis patients. Bacterial collagenase has been shown to be involved in lung hemorrhage induced by various infectious agents. According to Leptospira whole genome study, colA, a gene suggested to code for bacterial collagenase has been identified. We investigated colA gene expression in lung tissues of Leptospira infected hamsters. Golden Syrian Hamsters were injected intraperitoneally with Leptospira interrogans serovar Pyrogenes. The hamsters were sacrificed on days 3, 5 and 7 post-infection and lung tissues were collected for histological examination and RNA extraction. Lung pathologies including atelectasis and hemorrhage were observed. Expression of colA gene in lung tissues was demonstrated by both RT-PCR and real time PCR. In addition, ColA protein was cloned and the purified protein could react with sera from leptospirosis patients. Leptospira ColA protein may play a role in Leptospira survival or pathogenesis in vivo. Its reaction with leptospirosis sera suggests that this protein is immunogenic and could be another candidate for vaccine development.

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1. Introduction

Leptospirosis is the worldwide zoonosis caused by the spirochete bacteria in Genus Leptospira. This infectious disease is an important public health problem in both developing and industrialized countries. Humans acquire the disease by direct exposure to Leptospira or contact with soil or water contaminated with this organism. Leptospirosis patients present with a wide spectrum of symptoms ranging from asymptomatic or a flu-like syndrome to the severe disease with multi-organ failure. Weil’s disease, the severe form of leptospirosis is manifested by jaundice and renal failure. About 5–10% of leptospirosis patients exhibit severe manifestations and the overall mortality rate is 1–5% (Dolhnikoff et al. 2007a,b; Gouveia et al. 2008).

Although a number of Leptospira components have been identified, molecular mechanisms underlying pathologies observed in leptospirosis are still not well understood. Leptospirosis patients with different degrees of disease severity may acquire different numbers of organisms. In addition, patients with severe disease may be infected with high-virulence Leptospira. Host genetic background and immune response have also been proposed to involve in different degrees of disease severity.

Several Leptospira components such as hemolysin, lipopolysaccharide, glycolipoprotein, peptidoglycan, heat shock proteins, and flagellin have been identified and suggested to be involved in pathogenesis (Li et al. 2000; Nally et al. 2001; Picardeau et al. 2001; Lee et al. 2002; Lin et al. 2004; Branger et al. 2005; Hauk et al. 2005; Nally et al. 2005; Zhang et al. 2005; Matsunaga et al. 2007). Although Weil's disease is known to be the major cause of death of leptospirosis patients, pulmonary hemorrhage has been increasingly reported as an important clinical manifestation of leptospirosis (Chierakul et al. 2008; Dall’Antonia et al. 2008; Yang et al. 2009). However, molecular mechanisms underlying lung pathologies are not well understood.

Collagen is the fibrous structural protein that supports internal organs. Collagenase is an enzyme which breaks the peptide bond of collagen. The destruction of collagen by collagenase can be a cause of tissue damage. Several bacterial collagenases have been identified and suggested to be involved in tissue damage (Watanabe 2004).

Collagenase from Clostridium histolyticum induced lung hemorrhage when applied on surface of the dog lung or when injected intratracheally into mice (Vargaftig et al. 1976). In addition,
clostridium collagenase was shown to hydrolyze collagens type III and IV, the constituents of the tunica intima and media of blood vessels. This suggested that the hemorrhage could be induced by the collagenase activity (Hara-Kudo et al., 2008). Alveolar hemorrhage was frequently observed in patients with *Pseudomonas aeruginosa* sepsis. It has been shown that *P. aeruginosa* collagenase induced pulmonary and abdominal hemorrhage in mice (Baskerville et al. 1986; Diener et al. 1973). Collagenases from other bacteria such as *Serratia marcescens*, *Bacteroides fragilis* and *Pep-tostreptococcus magnus* were involved in pulmonary disorders or tissue destruction (Robertson et al. 1974; Lyerly and Kregger 1983; Krepel et al. 1992).

Degradation of host tissues by bacterial collagenase may benefit the organisms by facilitating colonization, spreading, invasion and immune evasion. Amino acids released from collagen degradation can be a nutrient source for bacterial growth.

Collagen degradation can be a direct mechanism in tissue damage or may promote further host immune response resulting in tissue destruction by host response (Harrington 1996). Collagenase from *Setaria cervi* has been studied as a vaccine candidate for human lymphatic filariasis. Injection of this collagenase induced strong anti-collagenase antibody and reduced parasite burden in challenged animals (Pokharel et al. 2006). This suggested that collagenase could be a target for vaccine development.

According to the *Leptospira* whole genome study, *coloA* (LA0872), a gene coding for collagenase, has been identified (Ren et al. 2003). However, *in vivo* expression of this gene has not been reported. In this study, we investigated whether *coloA* gene is expressed in lungs of hamsters infected with pathogenic *Leptospira*. In addition, *coloA* gene was cloned and protein was purified. The *coloA* gene product was investigated whether it could react with leptospirosis serum.

### 2. Materials and methods

#### 2.1. Hamster injection

The study was conducted under the approval of the Ethical Research Committee, Faculty of Medicine, Chulalongkorn University. Golden Syrian Hamsters (*Mesocricetus auratus*), aged 4–6 weeks were injected with 10⁸ *L. interrogans* serovar Pyrogenes, the isolate previously shown to induce pathologies in hamsters (Praditponsilpa et al. 2006). Hamsters were killed on days 3, 5, and 7 after infection. Lungs were collected in 10% formalin for pathological examination and in RNA stabilization solution (Invitrogen, CA) for RNA extraction. Four hamsters were used for each time-point.

#### 2.2. Tissue pathological study

Lung tissues fixed in 10% neutral buffered formalin were processed for paraffin embedding. Sections were cut at 3–5 µm in thickness and stained with hematoxylin and eosin on glass slides. The slides were reviewed by a pathologist under light microscope.

#### 2.3. RNA extraction and RT-PCR

Total RNA was extracted from 0.01 g of lung tissues using TRIzol reagent (Invitrogen) according to the manufacturer instruction. One microgram of RNA was treated with RNase-free DNase I (Ferments, MD) for residual DNA removal before using in cDNA synthesis. cDNA synthesis was performed by using random hexamer and M-MuLV reverse transcriptase (Ferments) at 42°C for 60 min.

Complementary DNA was amplified with primers specific to 16S rRNA (Merien et al. 1992) and *coloA* (LA0872) gene. The thermal cycling protocols were as follows: 94°C for 1 min, 55°C for 1 min and 72°C for 1 min for 30 cycles for 16S rRNA and 40 cycles for *coloA* gene. PCR products were visualized on 1.5% agarose gel with ethidium bromide staining.

#### 2.4. Real-time PCR for analysis of collagenase levels

The relative expression level of *coloA* to 16S rRNA was determined using the real-time quantitative SYBR-PCR. Reaction volumes of 25 µl consisted of the following components: 2.5 µl of cDNA template, 12.5 µl of 2× platinum qPCR supermix UDG (Invitrogen), and 500 nmol of each primer. The thermal profiles for 16S rRNA and *coloA* gene detection were as follows: an initial denaturation step of 50°C 2 min hold, 95°C 2 min hold, followed by 40 cycles of 95°C 15 s, 55°C 30 s, and 72°C 30 s. The data were analyzed by comparative threshold method in order to compare between the expression level of *coloA* gene in infected and in uninfected lungs tissues.

#### 2.5. *coloA* gene cloning

Full-length of *coloA* gene was amplified. The PCR product was then purified and ligated into pET100/D–TOPO (Invitrogen) plasmid according to the manufacturer’s instruction. The obtained sequence was compared with nucleotide sequence of *Leptospira colo* gene (LA0872) of *L. interrogans* serovar Copenhageni reported in NCBI (gi 45602555). The plasmid containing *colo* gene was transformed into C43 (DE3) *Escherichia coli*, the strain recommended for transformation of the gene for toxic protein.

#### 2.6. *coloA* protein purification

The C43 (DE3) *E. coli* containing *colo* gene was grown and protein expression was induced by 1 mM IPTG for 4 h. Bacteria were lysed by lysozyme treatment and sonication. *Colo* protein was purified from bacterial lysate using Protino® Ni–TED resin (Macherey-Nagel, Germany) according to manufacturer’s recommendation.

#### 2.7. *ColoA* protein reactivity against leptospirosis sera

Purified Colo protein was concentrated and one microgram of protein was subjected to 8% SDS-PAGE. Protein was transferred onto nitrocellulose membrane. The blot was blocked with 10% non-fat milk followed by incubating with 1:200 dilution of sera from patients confirmed to have leptospirosis by MAT (Microscopic agglutination test) and IHA (IgM). Anti-human IgG conjugated with horse radish peroxidase was then added. The protein band was visualized ECL Prime Western Blot Detection reagent (Amersham).

### 3. Results

#### 3.1. Lung pathology examination

The pathologic changes in lungs included vascular congestion, pulmonary hemorrhage and atelectasis. On day 3, three hamsters demonstrated mild to moderate pulmonary hemorrhage, all of which also had mild vascular congestion. However, lung tissue from a hamster displayed unremarkable findings. On day 5, all 4 hamsters presented with mild vascular congestion. Severe pulmonary hemorrhage was observed in 2 hamsters and mild hemorrhage was seen in one. On day 7, among four hamsters, one with mild, two with moderate and one with severe pulmonary hemorrhage were observed. Also, mild vascular congestion was seen in lung tissues from all four hamsters and atelectasis was detected in tissue from one hamster. Fig. 1B–D demonstrated the representatives of lung pathologies observed in hamsters sacrificed on days 3, 5 and 7, respectively. No significant pathological change was observed in lung tissues from non-infected hamsters (Fig. 1A).
Atelectasis and shown added; rRNA 1, Lanes 270 W.

3.2. colA gene expression in hamsters infected with pathogenic Leptospira

RT-PCR for 16S rRNA expression was done to demonstrate the presence of Leptospira in lung tissues. As shown in Fig. 2A, 16S rRNA was detected in all samples from infected hamsters.

Both RT-PCR and real time RT-PCR using the same primers were done to demonstrate colA gene expression. RT-PCR demonstrated colA gene expression in three out of four hamsters from each time-point (Fig. 2B). No-reverse transcriptase controls (RT-) were shown in Fig. 2C. There was no PCR product detected when cDNA synthesized from uninfected hamsters were tested (data not shown).

colA gene expression was also observed by real time RT-PCR. The expression was detected in all lung tissues from hamsters infected with Leptospira. Since no colA or 16S rRNA expression was detected in uninfected tissues, colA gene expression in infected lung tissues was demonstrated as the ratio between Ct of colA and Ct of 16S rRNA. The colA gene expression in lung tissues was demonstrated in Fig. 3A. The average of expression level in samples from days 3, 5 and 7 post-infection was shown in Fig. 3B. Although colA gene expression was detected in all samples, the level was relatively lower on day 7 after infection.

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Fig. 1. Pathological examination of lung tissues from Leptospira infected hamsters. Lung tissue from a non infected hamster (A) showed no significant pathological change. Atelectasis characterized by collapse of alveolar spaces was observed in lung tissue of hamsters infected with pathogenic Leptospira 3 days post infection (B). At day 5 (C) and day 7 (D) post-infection, pulmonary hemorrhage characterized by extravasated red blood cells in alveolar spaces was demonstrated.

Fig. 2. 16S rRNA and colA gene expression in lung tissues from hamsters infected with pathogenic Leptospira. RT-PCR was performed to demonstrate leptospiral 16S rRNA (A) and colA (B) gene expression as described in Section 2. No RT-controls were shown in (C). M: 100-bp marker; N: negative control (PCR master mix with no cDNA added); P: positive control (cDNA of Leptospira interrogans serovar Pyrogenes); Lanes 1, 4, 7 and 10 were from 3 days after infection; Lanes 2, 5, 8 and 11 from 5 days and Lanes 3, 6, 9 and 12 from 7 days after infection.

Fig. 3. ColA gene expression in lung tissues from hamsters infected with pathogenic Leptospira demonstrated by real time RT-PCR. The level of colA gene expression was investigated by SYBR-green real time PCR as described in Section 2. The relative expression levels of colA in lungs of 12 hamsters sacrificed on days 3, 5, and 7 after infection was demonstrated by ratio of colA Ct/16S rRNA Ct (A). The average expression levels of each time-point were shown in (B).
Sequences of primers used in this study were shown in Table 1.

3.3. colA gene product reacted with leptospirosis serum

colA gene in C43 (DE3) E. coli was induced by IPTG and protein was purified as described previously. The presence of protein eluted from Protino® Ni-TED resin was shown in Fig. 4A. The molecular weight predicted from amino acid sequence is 101 kDa. The apparent molecular weight of purified ColA protein on SDS-PAGE gel is about 97 kDa which is close to the molecular weight predicted from amino acid sequences.

The reactivity of cloned ColA protein was investigated by Western blot as described in Section 2. One control serum (serum from healthy individual) and five sera from leptospirosis patients were tested. As shown in Fig. 4B, Lane 1, there was no band detected when the blot was probed with control serum. Fig. 4B, Lanes 2–6, blots were probed with patient sera which are MAT positive for antibodies to L. interrogans serovars Shermanni, Bratislava, Shermani and Australis, respectively. The band at apparent molecular weight 97 kDa was detected. Different intensity of bands obtained may depend on different amount of antibodies to ColA protein present in each patient serum.

4. Discussion

It still remains unknown why leptospirosis patients present with a wide range of clinical manifestations. Several leptospiral components have been identified and some of them have been suggested as possible virulence factors of Leptospira. We are interested in lung pathologies induced by Leptospira infection.

Several genes which are hemolysin candidates have been cloned and studied. SpHA is a sphingomyelinase C with high hemolytic activity on both human and sheep erythrocytes (Segers et al. 1990). SpHII was shown to be a core forming protein. It mediated cytotoxicity of sheep erythrocytes and other mammalian cells. Hap-1 (hemolysin-associated protein) or LipL32 has been identified as a Leptospira secreted protein with hemolytic activity for erythrocytes (Lee et al. 2000, 2002). However, it has been later shown that LipL32 is not required for infection by Leptospira (Murray et al. 2009). HlyX is another hemolysin whose hemolytic activity has been confirmed. In addition, HlyX and LipL32 synergistically induced hemolysis (Hauk et al. 2005). SpH2 was recently identified as a sphingomyelinase-like hemolysin. Its cytotoxic activity was shown in mouse lymphocytes, macrophage and human liver cells. In addition, SpH2-treated liver cells demonstrated apoptotic morphological features (Zhang et al. 2008). Moreover, Leptospira contain genes homology to animal genes which encode platelet activating factor, acetylhydrolase and von Willebrand factor type A domain. These proteins may be involved in lung hemorrhage by activating hemostasis pathways (Ren et al. 2003; Yang et al. 2009).

We have previously reported that LipL32 expression could be detected in kidneys, livers and lungs of infected hamsters (Lowanitchapat et al. 2008). However, it has been recently shown that LipL32 is not required for tissue localization and pathology induction in hamsters (Murray et al. 2009). In this study, we demonstrated that Leptospira collagenase gene was expressed in lung, one of the common target organs in Leptospira infection. Collagenase gene expression was detected since day 3 after infection. The expression level seemed to be higher in samples from day 3 and day 5 than from day 7. Pathological examination demonstrated that pulmonary hemorrhage was more severe in lung tissues on days 5 and 7 after infection than on day 3.

Leptospira collagenase may be able to cause pulmonary hemorrhage directly or promote other factors suggested to cause tissue destruction. It has been shown that Leptospira altered sodium transport capacity of alveolar epithelial cells. This change might increase the susceptibility to lung injury (Andrade et al. 2007). In addition, immunohistochemical and immunofluorescent staining demonstrated inducible nitric oxide production in alveolar macrophage and immunoglobulin deposition in alveolar septum and alveolar space of a patient with leptospiral hemorrhagic respiratory failure (Yang and Hsu 2005). These suggested that lung pathologies could be induced directly by bacterial toxin, and/or by host immune response to infection.

Leptospira collagenase could be another promising protein for further study as another Leptospira virulence factor and as a vaccine candidate. In this study, we have cloned protein from Leptospira colA gene and demonstrated that this protein could react against leptospirosis sera. However, further study is required to demonstrate whether colA gene encodes protein with collagenase activity and the expression of this gene is involved in the progression of pulmonary hemorrhage.

Table 1
Sequences of primers used in this study.

<table>
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<tr>
<th>Primer name</th>
<th>Sequences (5’ → 3’)</th>
<th>Product size (bp)</th>
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<tr>
<td>16S rRNA-F</td>
<td>5′-CAAGTCACGGCGACTACCCAA-3′</td>
<td>290</td>
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<tr>
<td>16S rRNA-R</td>
<td>5′-CTTAACTGTCGCTCCGGAA-3′</td>
<td>209</td>
</tr>
<tr>
<td>Col F</td>
<td>5′-TCGACAAACGATACGTGGTAC-3′</td>
<td>168</td>
</tr>
<tr>
<td>Col R</td>
<td>5′-CGTCAACGATACCTCCATAC-3′</td>
<td>168</td>
</tr>
<tr>
<td>Full-ColA F</td>
<td>5′-CACCATCGAATAAGATATAGTAAG-3′</td>
<td>168</td>
</tr>
<tr>
<td>Full-ColA R</td>
<td>5′-GAAATTACGATCAGTATTCGG-3′</td>
<td>168</td>
</tr>
</tbody>
</table>

Sequencing primers

F1 5′-GGATATCCGATGGTTCCG-3′
F2 5′-CAAGTCACGGCGACTACCCAA-3′
F3 5′-ACAGACCTCAACAGCATTCC-3′
F4 5′-ACCATCGAATAAGATATAGTAAG-3′
R1 5′-GACCCGAACTGATCGATCTC-3′
R2 5′-CGTCAACGATACCTCCATAC-3′
R3 5′-TATGTATTTGACAAACACGCGG-3′
R4 5′-GAAATTACGATCAGTATTCGG-3′

Fig. 4. ColA protein reacted with leptospirosis sera. (A) ColA protein from E. coli lysate was purified using Protino® Ni-TED resin as mentioned in Section 2. The eluates were subjected to 8% SDS-PAGE and the gel was stained with Coomassie Brilliant Blue dye. Lanes 1–3 are 3 successive eluates from Protino® Ni-TED resin; M = molecular weight markers. The presence of ColA in eluates was indicated by an arrow. (B) ColA protein reaction against leptospirosis sera was demonstrated by Western blot as described in Section 2. Lane 1 was probed with control serum, Lanes 2–6 were probed with sera from three leptospirosis patients. The ColA protein band was indicated by an arrow. The apparent molecular weight of ColA protein detected was ~97 kDa. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
Acknowledgements

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References


