Identification of Collagenase as a Critical Virulence Factor for Invasiveness and Transmission of Pathogenic *Leptospira* Species

Kokouvi Kassegne,1,2,a Weilin Hu,1,2,a David M. Ojcius,3 Dexter Sun,4 Yumei Ge,1,2 Jinfang Zhao,5 X. Frank Yang,6 Lanjuan Li,1 and Jie Yan1,2

1Division of Basic Medical Microbiology, State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, First Affiliated Hospital, Zhejiang University School of Medicine, 2Department of Medical Microbiology and Parasitology, Zhejiang University School of Medicine, and 3Department of Clinical Laboratory, Zhejiang Traditional Chinese Medical Hospital, Hangzhou, China; 4Health Sciences Research Institute and School of Natural Sciences, University of California, Merced; 5Department of Neurology and Neuroscience, New York Presbyterian Hospital and Hospital for Special Surgery, Cornell University Weill Medical College, New York, and 6Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis

**Background.** Leptospirosis is a global zoonotic disease. Transmission of *Leptospira* from animals to humans occurs through contact with water contaminated with leptospire-containing urine of infected animals. However, the molecular basis for the invasiveness of *Leptospira* and transmission of leptospirosis remains unknown.

**Methods.** Activity of *Leptospira interrogans* strain Lai colA gene product (ColA) to hydrolyze different collagenic substrates was determined by spectrophotometry. Expression and secretion of ColA during infection were detected by reverse-transcription quantitative polymerase chain reaction and Western blot assay. The ColA gene–deleted (ΔcolA) and ColA gene–complemented (CΔcolA) mutants were generated to determine the roles of ColA in transcytosis in vitro and virulence in hamsters.

**Results.** Recombinant or native ColA hydrolyzed all the tested substrates in which type III collagen was the favorite substrate with 2.16 mg/mL K_m and 35.6 h⁻¹ K_cat values. Coincubation of the spirochete with HUVEC or HEK293 cells directly caused the significant elevation of ColA expression and secretion. Compared with wild-type strain, ΔcolA mutant displayed much-attenuated transcytosis through HEK293 and HUVEC monolayers, and less leptospires in blood, lung, liver, kidney and urine and 25-fold-decreased 50% lethal dose and milder histopathological injury in hamsters.

**Conclusions.** The product of colA gene is a collagenase as a crucial virulence factor in the invasiveness and transmission of *L. interrogans*.

**Keywords.** *Leptospira*; Collagenase; Invasiveness; Tissue injury; Transmission.

Leptospirosis caused by pathogenic *Leptospira* species is a global zoonotic infectious disease [1], especially in Southeast Asia and South America [2, 3]. In North America and Europe, leptospirosis is considered an emerging infectious disease because of frequent case reports and several outbreaks in recent years [4–7].

Many animals can serve as hosts of pathogenic *Leptospira* species, including dogs and livestock [8]. After being shed in urine of the infected animals, the spirochete survives for long periods of time in moist soil and natural bodies of water [8, 9]. After contact with the skin and mucosa of individuals, the leptospires in the contaminated soil or water invade the human body promptly to cause septicemia, followed by a rapid spread into lungs, liver, and kidneys to cause tissue injury [9]. Leptospirosis is clinically characterized by high fever, myalgia, hemorrhage, jaundice, renal impairment, and septic shock [10]. In the convalescence stage, patients also
discharge leptospires in the urine for about 1 week [8–10]. Until now, however, little has been known about the molecular basis for the powerful invasiveness and transmission of pathogenic *Leptospira* species.

Previous reports revealed that a collagenase of *Clostridium histolyticum* contributes to damage of cells and spread in tissues of hosts [11, 12]. Collagenases of *Clostridium perfringens*, *Vibrio parahaemolyticus*, and *Fusobacterium nucleatum* act as virulence factors for invasiveness and tissue injury [13–15]. These data indicate that bacterial collagenases play important roles in invasiveness and transmission during infection.

*Leptospira interrogans* is the most common genotype in pathogenic *Leptospira* species [1–7]. Several serogroups of *L. interrogans* are epidemic in China, but *L. interrogans* serogroup Icterohaemorrhagiae serovar Lai is responsible for leptospirosis in about 70% of patients [3, 16]. The chromosomal DNA of *L. interrogans* serovar Lai strain Lai includes a single *colA* gene that is predicted to encode a collagenase [17]. However, the collagenolytic activity and pathogenic role of product of the gene have not been characterized.

In the current study, we determined the distribution of *colA* gene in *L. interrogans* strains belonging to different serogroups that prevalent in China as well as the ability of the *colA* gene product of *L. interrogans* strain Lai to hydrolyze different collagenic substrates. Next, we investigated the expression and secretion of ColA protein in the spirochete during human renal epithelial and blood vessel endothelial cell infection. Finally, we generated a *colA* gene–deleted mutant to further evaluate the role of ColA protein in the invasiveness and transmission of the spirochete in cell monolayers and animals. The results of our study identify ColA as a novel and crucial virulence factor of *L. interrogans*.

**MATERIALS AND METHODS**

**Animal**

Male Syrian hamsters (mean weight ± standard deviation [SD], 25 ± 2 g per animal) were provided by the Experimental Animal Center of Zhejiang University. The animal experimental protocols were approved by the Ethics Committee for Animal Experiment of Zhejiang University.

**Leptospiral Strain, Cell Lines, and Culture**

Seven pathogenic *L. interrogans* strains and 2 nonpathogenic *Leptospira biflexa* strains belonging to different serogroups or serovars (see Supplementary Information) were cultivated at 28°C in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium [18]. A human renal tubular epithelial cell line (H442) and a human umbilical vein endothelial cell line (HUVEC) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) medium (Gibco), supplemented with 10% fetal calf serum (FCS; Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in an atmosphere of 5% carbon dioxide (CO2).

**Detection of colA Gene, Preparation of Recombinant and Native ColA Proteins, and Preparation of Antiserum and Immunoglobulin G**

See the Supplementary Information for details about detection of the *colA* gene in different leptospiral strains by polymerase chain reaction (PCR) and sequencing; preparation of the recombinant ColA (rColA) and native ColA proteins from *L. interrogans* strain Lai; and preparation of rabbit antiserum and immunoglobulin G (IgG) antibodies against rColA.

**Collagen Hydrolysis Test**

One milliliter of Calcium binding buffer (CBB) (0.4 mmol/L calcium chloride and 50 mmol/L Tris-HCl; pH 7.4) containing 5 mg of native bovine type I or II collagen, human type III or IV collagen (Sigma), or synthetic substrate Azocoll or Pz peptide (Sigma), was mixed with 50 or 100 μg of rColA or 100 μg of native ColA protein. The mixtures were incubated at 37°C for 5 hours. Hydrolysis of the substrates was measured by spectrophotometry at an optical density at 570 nm (collagen hydrolysis), 520 nm, or 320 nm (Azocoll or Pz peptide hydrolysis) [19–21]. In addition, collagenolytic ability of the rColA was checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 100 μg of rColA for hydrolysis with 5 mg of type III collagen and 200 μg of rColA for hydrolysis with 5 mg of type I, II, or IV collagen. Moreover, solutions of 0.2 mol/L sodium phosphate with 0.15 mol/L sodium chloride (pH 7.0) or 0.2 mol/L sodium borate with 0.15 mol/L sodium chloride (pH 7.2) were used to evaluate the influence of different buffers on hydrolytic activity of the rColA [19–21].

**Collagen Hydrolysis Inhibition Test**

The rColA or native ColA protein (100 μg) was pretreated with 5 μmol/L collagenase inhibitor I (Millipore) for a 30-minute incubation at 37°C in CBB buffer [22]. The ability of the ColA proteins before or after treatment with the inhibitor to hydrolyze the 4 collagens was determined as already described.

**Determination of Km and Kcat Values**

The collagen hydrolysis test demonstrated type III collagen as the preferred substrate of rColA and native ColA protein. To determine the enzyme kinetic parameters (Km and Kcat), 0.25, 0.5, 1, 2, 3, 4, or 5 mg of type III collagen was mixed with 100 μg of rColA in 1 mL of CBB buffer, and the hydrolysis of type III collagen was performed as already described. The Km and Kcat values of rColA to hydrolyze type III collagen were calculated by means of double reciprocal Lineweaver-Burk plot [23].

**Measurement of Transcription and Expression of colA Gene During Infection**

Freshly cultured *L. interrogans* strain Lai was collected by using 17200 g centrifugation at 15°C for 15 minutes. The harvested leptospires were counted with a Petroff-Hausser counting chamber (Fisher Scientific) under a dark-field microscope [24].
HUVEC and HEK293 cells (1 × 10^6 per well) were seeded in 6-well culture plates (Corning) for a 12-hour incubation in an atmosphere of 5% CO_2. After being washed with phosphate-buffered saline (PBS), the cell monolayers were infected with the spirochete (1 × 10^8) at 37°C for 0.5, 1, 2, 4, 8, 12 or 24 hours [25–27]. Media from the leptospire cell cocultures were collected by a 15-minute centrifugation at 17 200 g. The spirochete (1 × 10^8) was inoculated into the media or in 8% FCS-DMEM for incubation at 37°C for the same durations. On the other hand, the cocultures were treated with 0.05% sodium deoxycholate–PBS to lyse cells [28], followed by a short centrifugation at 500 g to remove cell debris. The supernatants as well as the leptospiral cultures in the different media were centrifuged at 17 200 g for 15 minutes (4°C) to precipitate leptospirases.

Total leptospiral RNA was extracted using TRIzol reagent (Sigma) and digestion with RNase-Free DNase (TaKaRa). Complementary DNA (cDNA) was synthesized from the total RNA by reverse transcription (RT) using a cDNA Synthesis Kit (TaKaRa). Using the cDNA as template, the colA messenger RNA (mRNA) was measured with real-time fluorescence quantitative PCR (qPCR) using a SYBR Ex-Taq Kit (TaKaRa). In the RT-qPCR, 16S RNA gene of the spirochete was used as the inner reference [29]. The RT-qPCR data were analyzed using the ΔΔCt model and a randomization test with REST 2005 software [30].

The HUVEC or HEK293 monolayer (1 × 10^6 per well) was infected with the spirochete (1 × 10^8) for 1, 2, 4, 8, or 12 hours. Incubation of the spirochete in the media from the leptospire cell cocultures or in 8% FCS-DMEM, and lysis of the cells and precipitation of leptospirases were performed as already described. The leptospirases were ultrasonically broken and then centrifuged at 500 g for 15 minutes. The supernatants were collected to detect protein concentrations using a Protein Concentration Assay Kit (Bio-Rad). Using rabbit anti-rColA IgG as the primary antibody and horseradish peroxidase–conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) as the secondary antibody, the ColA in the protein specimens was detected with anti-rabbit IgG (Jackson ImmunoResearch) as the secondary antibody. ColA protein in the protein specimens was detected by means of Western blot assay. The immunoblotting signals were quantified as described. In the assay, a leptospiral cytoplasmic protein (FiLy) and a secretory hemolysin (Sph2) were used as the controls [32, 33].

Generation and Identification of colA Gene–Deleted mutant and colA Gene–Complemented Mutants

See the Supplementary Information for details about the generation and identification of the colA gene–deleted (ΔcolA) and colA gene–complemented (CΔcolA) mutants.

Transwell migration Assay

HUVEC or HEK293 cells (1 × 10^6 cells per well) were seeded in the upper compartment of a 12-well Transwell plate (Corning) and then incubated for 48 hours in an atmosphere of 5% CO_2 at 37°C to form tight cell monolayers. Transendothelial or epithelial electric resistance (TEER) of the cell monolayers was monitored using a Millicell electric resistance indicator (Millipore); TEER values >200 Ω/cm² indicated the integrity of cell monolayer [34]. The HUVEC or HEK293 cell monolayers was infected with L. interrogans strain Lai (1 × 10^8) for 1, 2, 4, 8, 12, 24, or 48 hours at 37°C. The leptospirases that transmigrated from the upper into lower compartments were counted with Petroff-Hauser counting chamber (Fisher Scientific) under a dark-field microscope [24]. In the assay, the spirochete (1 × 10^8) through the upper compartments without cells was used as the controls.

Determination of ColA Virulence in Hamsters

Syrian hamsters were intraperitoneally injected with 10^4, 10^5, 10^6, or 10^7 leptospirases of the ΔcolA or CΔcolA mutant or wild-type L. interrogans strain Lai, with 8 animals used per group [35]. Eight negative control animals were intraperitoneally injected with EMJH medium. The animals were monitored twice daily, and their survival was recorded within 14 days after challenge to calculate the 50% lethal dose using Warren probit analysis [28]. In addition, hamsters (n = 6) were infected with each of the mutant and wild-type strains, as already described. Lung, liver, and kidney specimens were collected on days 3 and 7 after challenge for histopathological examination after hema-toxylin-eosin staining.

Determination of ColA Secretion During Infection

HUVEC or HEK293 cell monolayer (1 × 10^6 per well) was infected with L. interrogans strain Lai (1 × 10^8) for 1, 2, 4, 8, or 12 hours. After lysis of the cells, the supernatants were harvested by using 17 200 g centrifugation at 4°C for 15 minutes. Meanwhile, the supernatant from the spirochete (1 × 10^8) in EMJH medium or incubated in 8% FCS-DMEM for the indicated times was also collected by centrifugation, as described. Total proteins in all the supernatants were extracted by trichloroacetic acid precipitation [32]. With rabbit anti-rColA IgG used as the primary antibody and horseradish peroxidase–conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) as the secondary antibody, ColA protein in the protein specimens was detected by means of Western blot assay. The immunoblotting signals were quantified as described. In the assay, a leptospiral cytoplasmic protein (FiLy) and a secretory hemolysin (Sph2) were used as the controls [32, 33].
Distribution of colA gene, preparation of recombinant ColA (rColA) and native ColA protein and characterization of the colA gene–deleted (ΔcolA) and colA gene–complemented (CΔcolA) mutants. A, Distribution of colA gene in different pathogenic Leptospira interrogans and nonpathogenic Leptospira biflexa strains. Lane M, DNA marker. Lane 1, Blank control. Lanes 2–8, Amplicons of colA gene (2667 base pairs [bp]) from L. interrogans serovar Lai strain Lai, serovar Grippotyphosa strain Lin-6, serovar Autumnalis strain Lin-4, serovar Pomona strain Luo, serovar Hebdomadis strain P7, serovar Australis strain 65-9, and serovar Canicola strain Lin, respectively. Lanes 9–10, Negative polymerase chain reaction (PCR) results for colA gene detection of L. biflexa serovar Patoc strain Patoc-1 and serovar Adamana strain CH-11. B, Expression of rColA and purification of rColA and native ColA protein. Lane M, Protein marker. Lane 1, Blank control (wild-type pET42a). Lanes 2–3, Expressed and purified rColA protein, respectively. Lane 4, Native ColA protein extracted using immunoprecipitation resin. C, Identification of ΔcolA and CΔcolA mutants by PCR. Lane M, DNA marker. Lane 1, Blank control. Lane 2, Amplicon (2589 bp) of the signal peptide–absent colA gene from L. interrogans strain Lai for recombinant expression. Lane 3, Amplicon (3569 bp) of the colA-kan-colA segment (3329 bp) plus 2 extending regions (120 bp each) for characterization of the ΔcolA mutant. Lane 4, Amplicon (5314 bp) of the 5′ arm-colA-spc-3′ arm (5074 bp) plus 2 extending regions (120 bp each) for characterization of the CΔcolA mutant. D, Schematic diagram of the sequencing result for ΔcolA mutant. Positions of PCR primers used are marked below. E, Schematic diagram of the sequencing result of CΔcolA mutant. Positions of PCR primers used are marked below.
examination of leptospires in tissues after silver staining [36]. In addition, 5 mg of the tissue specimens was homogenized in an ice bath and then added with 5 mL of PBS. The mixtures were centrifuged at 300 g for 10 minutes (4°C) to collect supernatants. Next, 0.1 mL of the supernatant or blood specimens was inoculated into 1 mL of Korthof medium for a 24-hour incubation and then spread on EMJH agar plates for a 3-week incubation to count colony-forming units [36]. The urine specimens were centrifuged at 250 g for 5 minutes to remove particulate matters, followed by a 15-minute centrifugation at 17 200 g to precipitate leptospires. For counting leptospires, the pellets were suspended at the 1:1 or 1:100 volume ratio of PBS to the urine specimens, as described above [24].

**Statistical Analysis**
Data from a minimum of 3 experiments were averaged and presented as means ± SDs. Statistical significance was determined with 1-way analysis of variance followed by the Dunnett multiple-comparison test, with differences considered significant at P < .05.

**RESULTS**

**Sequencing and Expression of colA Gene**
All the tested 7 pathogenic*L. interrogans* strains, but not the 2 nonpathogenic*L. biflexa* strains, have the colA gene (Figure 1A) with 99.4%–100% amino acid sequence identity compared with that in the report (data not shown) [17]. The*Escherichia coli* BL21DE3pET42a-colA expressed rColA, and the purified rColA or native ColA protein showed a single band in gel after electrophoresis (Figure 1B).

**Characterization of ΔcolA and ΔΔcolA Mutants**
The ΔcolA and ΔΔcolA mutants could grow persistently in EMJH medium with typical shape and motility and similar growth kinetics compared with the wild-type strain (data not shown). The PCR (Figure 1C) and sequencing data (Figure 1D)[18–20].

![Figure 2](https://academic.oup.com/jid/article-abstract/209/7/1105/878582)

**Figure 2.** Collagenase activity of recombinant ColA (rColA) and native ColA proteins from*Leptospira interrogans* strain Lai. A, Ability of rColA and native ColA proteins to hydrolyze different collagenase substrates, determined with spectrophotometry. Bars show means ± standard deviations (SDs) of 3 independent experiments. In this test, CBB buffer was used. The hydrolysis of type I, II, III, or IV collagen and synthetic Azocoll or Pz peptide substrate was measured by spectrophotometry at OD570 (optical density at 570 nm), OD520 or OD320, respectively. *P < .05 (vs hydrolytic activity of 50 µg of rColA). B, Attenuated collagenolytic ability of rColA and native ColA proteins after treatment with collagenase inhibitor I. Bars show means ± SDs of 3 independent experiments. The buffer and spectrophotometric examination were as in A. *P < .05 (vs hydrolytic activity of rColA and native ColA protein before treatment with collagenase inhibitor I). C, Ability of rColA to hydrolyse native collagens, demonstrated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. In this test, 100 µg of rColA was used for 5 mg of type III collagen hydrolysis, and 200 µg of rColA for 5 mg of type I, II, or IV collagen hydrolysis and CBB buffer. Lane M, Protein marker. Lanes C1–C4, Type I, II, III, or IV collagen before hydrolysis. Lanes T1–T4, Hydrolysis of type I, II, III, or IV collagen after incubation with rColA. D, Influence of different reactive conditions on collagenase activity of rColA. Bars show means ± SDs of 3 independent experiments. The hydrolysis of type I-IV collagens, synthetic Azocoll and Pz peptide substrate was measured by spectrophotometry at OD570, OD520 and OD320, respectively. *P < .05 (vs hydrolytic activity of rColA in the CBB buffer). CaCl2, calcium chloride; NaCl, sodium chloride. E, Km and Kcat values of rColA hydrolyzing type III collagen in CBB buffer. In this assay, 100 µg of rColA and different concentrations of type III collagen (0.25, 0.5, 1, 2, 4, or 5 mg) were used. The hydrolysis of type III collagen was measured by spectrophotometry at OD570. [S] = substrates, V = velocity of substrate hydrolysis. The image showed the ability of rColA to hydrolyze substrate with different concentrations per hour.
Figure 3. Increase in colA gene expression and ColA protein secretion during infection. 

A. Increase in *Leptospira interrogans* strain Lai colA mRNA levels during infection of HUVEC or HEK293 cells for the indicated times. Bars show means ± standard deviations (SDs) of 3 independent experiments. The values at 0 hour indicate the colA messenger RNA (mRNA) levels in the spirochete before infection or incubation, set as 1.0. *P < .05 (vs colA mRNA levels in the spirochete before infection or incubation [0 hour]); †P < .05 (vs the colA mRNA levels in the spirochete incubated in Dulbecco’s modified Eagle’s medium (DMEM) or in media from leptospire cell cocultures for the indicated times). 

B. Increase in ColA protein expression in *L. interrogans* strain Lai during infection of HUVEC or HEK293 cells for the indicated times. The immunoblotting results at 0 hour indicate the ColA protein expression in the spirochete before infection or incubation. ColA-1 indicates the expressed ColA protein in the spirochete during infection of HUVEC or HEK293 cells; ColA-2, expression of ColA protein in the spirochete during incubation in the media from leptospire-HUVEC or leptospire-HEK293 cocultures; ColA-3, expression of ColA protein in the spirochete during incubation in 2.5% fetal calf serum (FCS)-DMEM medium; LipL41 is a leptospiral outer membrane lipoprotein used as the control.

C. Quantification of immunoblotting bands (by gray scale determination) reflecting ColA protein expression levels during infection of host cells for the indicated times (statistical data from experiments as shown in B). Bars show means ± SDs of 3 independent experiments. Gray scale values of immunoblotting bands reflect the ColA expression levels in *L. interrogans* strain Lai before infection or incubation (0 hour). *P < .05 (vs ColA expression levels [gray scale values] in the spirochete before infection or incubation [0 hour]).

D. Enhancement of ColA secretion of *L. interrogans* strain Lai during infection of HUVEC or HEK293 cells for the indicated times. The immunoblotting results at 0 hour indicate no secretory ColA protein in the supernatant from culture of the spirochete in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium. FliY is a leptospiral cytoplasmic protein and Sph2 a leptospiral secretory hemolysin, used as controls. The DMEM lane indicates the ColA protein secretion in the supernatants from cultures of the spirochete in 2.5% FCS-DMEM medium. 

E. Quantification of immunoblotting bands (by gray scale determination) reflecting the secretion of ColA protein during infection of host cells for the indicated times (statistical data from experiments such as shown in D). Bars show means ± SDs of 3 independent experiments. The values at 0 hour indicate the gray scale values, reflecting no secretion of ColA protein in the supernatant from culture of *L. interrogans* strain Lai in EMJH medium. *P < .05 (vs the secretion level [gray scale value] of ColA protein in the supernatant from culture of the spirochete in EMJH medium [0 hour]).
The data suggest that the collagenase encoded by the colA gene is required by L. interrogans during infection and that direct leptospire cell interaction causes the elevation of ColA protein expression.

Enhancement of ColA Secretion During Infection
The ColA protein of wild-type L. interrogans strain Lai was undetectable in EMJH medium and the incubation of the spirochete in DMEM medium also did not cause the secretion of ColA protein (Figure 3D and 3E). When the spirochete was incubated with HUVEC or HEK293 cells, the ColA protein was detectable in the supernatants from the cocultures and the secretion levels of ColA protein were significantly elevated in a time-dependent manner (Figure 3D and 3E). The findings suggest that the collagenase encoded by colA gene of L. interrogans plays a direct role during infection and that leptospire cell interaction induces the secretion of collagenase.

Attenuated Transcytosis of ΔcolA Mutant
The Transwell migration assay demonstrated that transcytosis of the ΔcolA mutant through the HUVEC or HEK293 cell monolayers was significantly attenuated compared with the ΔcolA mutant or wild-type L. interrogans strain Lai (Figure 4A). The high TEER values (205–216 Ω/cm²) of the 2 cell monolayers in the whole infection process indicated that the integrity of cell monolayers was not damaged (Figure 4B) [34]. The data suggest that the ColA protein is a powerful invasive enzyme of L. interrogans.

Reduced Leptospire Loading in Tissues and Discharge in Urine of ΔcolA Mutant
There were noticeably fewer leptospires in the lung, liver, and kidney tissues from the ΔcolA mutant–infected hamsters than from the hamsters infected with the ΔcolA mutant or wild-type L. interrogans strain Lai (Figure 5A). Similarly, significant fewer leptomisal colonies were isolated from the lung, liver, kidney, or blood specimens from the ΔcolA mutant–infected...
hamsters than from the CΔcolA mutant or wild-type strain–infected hamsters (Figure 5B). In particular, compared with hamsters infected with the CΔcolA mutant or wild-type strain, those infected with the ΔcolA mutant had many fewer leptospires in urine (Figure 5D and 5E). The data indicate that the collagenase expressed by the colA gene plays an important role in the dissemination in vivo and discharge into urine of L. interrogans during the infection of hosts.

Attenuated Virulence of ΔcolA Mutant in Hamsters

The 50% lethal dose in hamsters within 14 days after challenge was $2.4 \times 10^7$ leptospires for the ΔcolA mutant, $1.0 \times 10^6$ leptospires for the CΔcolA mutant, and $0.94 \times 10^6$ leptospires for wild-type L. interrogans strain Lai. When $1 \times 10^6$ leptospires were used as the challenge dose per animal, the wild-type strain or CΔcolA mutant caused more serious pathological injury in the lung, liver, and kidney of the infected hamsters than the ΔcolA mutant (Figure 6). The data indicate that the collagenase encoded by colA gene contributes to the virulence of L. interrogans.

DISCUSSION

Virulence of bacterial pathogens is dependent on their invasiveness and toxins. Invasiveness reflects the ability of bacteria
to adhere to host cells, penetrate through the epithelial barrier, invade the bloodstream, and spread into different tissues and organs [37]. Collagenase is a common invasive enzyme, playing an important role in the invasiveness of bacteria [11, 38, 39]. Pathogenic Leptospira species have powerful invasiveness, enabling the pathogen to quickly invade the human body and spread into internal organs [3, 7, 36, 40]. Previous studies revealed that pathogenic Leptospira strains, but not nonpathogenic strains, could transmigrate across vascular endothelial and renal epithelial cell monolayers [34]. These data indicate that invasiveness is required by pathogenic Leptospira species to cause disease.

Collagens serve as the major structural component of nearly all mammal tissues [41–43]. Type I collagen exists in the extracellular matrix of cells in the corium layer, and type II collagen is only found in cartilage and vitreum [41]. Type III collagen is abundant in the vessel walls, corium, lung, heart, and gastrointestinal tract [42], and type IV collagen is distributed in the vessel wall as a component of the basal membrane [43]. Thus, type I, III, and IV collagens participate in forming epithelial and endothelial barriers against the invasion and dissemination of pathogens, including L. interrogans.

No collagenase-encoding genes could be found in the genomic DNA of nonpathogenic L. biflexa [44]. However, several

Figure 6. Histopathological damage in hamsters infected with colA gene-deleted (ΔcolA) or colA gene-complemented (ΔΔcolA) mutant or wild-type Leptospira interrogans strain Lai. Lung, liver, and kidney specimens of hamsters were collected on day 7 after challenge with the ΔcolA or ΔΔcolA mutant or wild-type L. interrogans strain Lai. Serious congestion and multiple focal necrosis of nephric tubular epithelia in kidney, visible hemorrhaging, and inflammatory cell infiltration in lung, and extensive hepatocyte necrosis in liver occurred in the hamsters infected with the ΔΔcolA mutant or wild-type strain (10^6 leptospires per animal). Conversely, mild cellular edema in kidney, inflammatory cell infiltration in lung, and slight granular degeneration in liver occurred in the hamsters infected with the same amount (10^6 leptospires) of the ΔcolA mutant.
Pathogenic *Leptospira* species have been shown to possess a single putative collagenase-encoding gene (*colA*) [16, 45, 46], but collagenase activity of the gene product has never been characterized. We also found that all 7 tested strains of pathogenic *L. interrogans*, but not the 2 *L. biflexa* strains, had the *colA* gene with higher sequence conservation (99.4%–100% identities), suggesting that the *colA* gene is required by *L. interrogans* during infection of hosts [47].

In the current study, we demonstrated that both the rColA and native ColA proteins expressed by the *colA* gene of *L. interrogans* strain Lai can efficiently hydrolyze all the tested native collagens and synthetic collagenlike substrates, in which type III collagen is the preferred substrate. When the spirochete was incubated with HUVEC or HEK293 cells, the expression and secretion of ColA protein were significantly increased. However, the spirochete incubated in DMEM medium or in the media from leptospire cell cocultures caused only a small *colA* mRNA level elevation, but no secretion of ColA protein could be detected. These data indicate that the product of *colA* gene is a collagenase of *L. interrogans* and direct leptospire cell interaction triggers the up-regulation of collagenase expression and secretion.

Pathogenic *Leptospira* species have not been found to produce typical exotoxins, and the toxicity of leptospiral lipopolysaccharide is much lower than that of *E. coli* [32, 36]. Therefore, the powerful invasiveness is considered the major agent responsible for pathogenicity of *Leptospira* [3, 28, 48]. The results of Transwell migration assay revealed the significantly attenuated ability of the *colA* gene–deleted (Δ*colA*) mutant to transmigrate through HUVEC and HEK293 cell monolayers compared with the wild-type strain. The animal test also demonstrated that the number of leptospires in blood specimens from the hamsters at early stages of infection with the Δ*colA* mutant was visibly lower than that from hamsters infected with the wild-type strain. More importantly, significantly fewer leptospires were recovered from the lung, liver, and kidney specimens of the Δ*colA* mutant–infected hamsters than from the wild-type strain–infected hamsters. The Δ*colA* mutant also induced less pathologic injury in tissues of the 3 organs than the wild-type strain. These results indicate that the product of *colA* gene is an important virulence factor of *L. interrogans* during infection owing to its invasiveness-related collagenase activity.

Transmission of leptospires from animals to humans usually occurs indirectly through contact with soil or water contaminated by urine from the leptospire-infected animal [6, 7, 9]. Unlike the temporal leptospire discharge in urine of patients, the leptospire-infected animals persistently shed leptospires from urine for long periods of time into water and soil to form an infection source for leptospirosis [8, 9, 36]. Compared with the wild-type strain, much lower numbers of the Δ*colA* mutant was found in the urine of infected hamsters. Taken together, our findings proved that the *colA* gene of *L. interrogans* encodes a collagenase that contributes to the virulence of the spirochete and the transmission of leptospirosis.

**Supplementary Data**

**Supplementary materials** are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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**References**


