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

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**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 (*Δ*colA) and *colA* gene–complemented (*CΔ*colA) 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*<sub>m</sub> and 35.6 h<sup>−1</sup> *K*<sub>cat</sub> 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, *Δ*colA 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.
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 paraheamoliticus, 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 (Htu293) 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 collagen 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 17 200 g centrifugation at 15°C for 15 minutes. The harvested leptospires were counted with a Petroff-Hauser 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% CO₂. 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 leptosporal cultures in the different media were centrifuged at 17 200 g for 15 minutes (4°C) to precipitate leptospires.

Total leptosporal 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 leptosporal cell cocultures or in 8% FCS-DMEM, and lysis of the cells and precipitation of leptospires were performed as already described. The leptospires 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, ColA protein in the protein specimens was detected with anti-rabbit IgG (Jackson ImmunoResearch) as the secondary antibody. The immunoblotting signals were quantified as described. In the assay, a leptospiral cytoplasmic protein (FltY) 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% CO₂ 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 × 10⁵) for 1, 2, 4, 8, 12, 24, or 48 hours at 37°C. The leptospires that transmigrated from the upper into lower compartments were counted with Petroff-Haussler counting chamber (Fisher Scientific) under a dark-field microscope [24]. In the assay, the spirochete (1 × 10⁸) through the upper compartments without cells was used as the controls.

**Determination of ColA Virulence in Hamsters**

Syrian hamsters were intraperitoneally injected with 10⁴, 10⁵, 10⁶, 10⁷, or 10⁸ leptospires 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 hematoxylin–eosin staining.

**Determining the Role of ColA in Leptospiral Invasiveness in Hamsters**

Syrian hamsters (n = 54) were challenged with 1 × 10⁸ leptospires of the ΔcolA or CΔcolA mutant or wild-type L. interrogans strain Lai, as described above. Heart blood specimens were collected on day 1 or 3, lung, liver, and kidney on day 3 or 7, and urine specimens on day 7 or 14 after infection [36]. The sections of lung, liver, and kidney specimens were prepared for

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Figure 1. 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 Gripotyphosa 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 5′-arm-colA-spc-3′ arm (5074 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 CΔcolA Mutants
The ΔcolA and CΔcolA 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)
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 the L. interrogans ΔcolA mutant and the colA gene complementation in the CΔcolA mutant. The RT-qPCR and Western blot assays also proved the absence or presence of colA mRNA and ColA protein in the ΔcolA or CΔcolA mutant (data not shown).

**Collagenase Activity of rColA and Native ColA Protein**

Both the rColA and native ColA proteins from L. interrogans strain Lai had the similar ability to hydrolyze all the 4 native collagens and 2 synthetic substrates, but the type III collagen was the preferred substrate, with the highest hydrolytic ratio (Figure 2A). However, the collagenolytic activity was blocked by collagenase inhibitor I (Figure 2B). The ability of rColA to hydrolyze 4 collagens was shown in Figure 2C. In the CBB buffer, the rColA displayed a higher collagenolytic ability than in the other 2 buffers (Figure 2D). The Km and Kcat values of rColA for hydrolysis of type III collagen in CBB buffer were 2.16 mg/mL and 35.6 hours⁻¹, respectively (Figure 2E).

**Elevation of ColA Expression During Infection**

Wild-type L. interrogans strain Lai in DMEM medium or in the media from leptospire cell cocultures showed a small colA mRNA level elevation compared with that in EMJH medium, but a rapid and dramatic colA mRNA level elevation in the spirochete was found during infection of HUVEC or HEK293 cells (Figure 3A). The ColA protein expression in the spirochete from EMJH medium was relatively low, and the incubation of the spirochete with DMEM medium or the media from leptospire cell cocultures did not up-regulate the expression of ColA protein (Figure 3B and 3C). When the spirochete was incubated with the two cell types, the expression levels of ColA protein were significantly elevated in a time dependent manner (Figure 3B and 3C). The data suggest that 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 CΔcolA 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 CΔcolA mutant or wild-type L. interrogans strain Lai (Figure 5A). Similarly, significant fewer leptospiral colonies were isolated from the lung, liver, kidney, or blood specimens from the ΔcolA mutant–infected hamsters.
hamsters than from the ΔcolA mutant or wild-type strain–infected hamsters (Figure 5B and 5C). In particular, compared with hamsters infected with ΔcolA or colA gene–complemented (ΔcolA) mutant or wild-type L. interrogans strain Lai for the indicated times. The leptospiries in the tissue specimens were stained with a silver staining method. Arrows indicate leptospiries in the tissues. B, Colony counts in lung, liver, and kidney specimens from hamsters infected with ΔcolA or ΔcolA mutant or wild-type L. interrogans strain Lai for the indicated times. Bars show means ± standard deviations (SDs) of colonies from 3 separate samples of ≥3 animals. *P < .05 (vs colony numbers in the lung, liver, and kidney specimens of the hamsters infected with the ΔcolA mutant or wild-type strain). C, Colony counts in blood specimens from hamsters infected with ΔcolA or ΔcolA mutant or wild-type L. interrogans strain Lai for the indicated times. Bars show means ± SDs of colonies from 3 separate samples of ≥3 animals. *P < .05 (vs colony numbers in the peripheral blood specimens of the hamsters infected with the ΔcolA mutant or wild-type strain). D, Leptospiries in urine specimens of the hamsters infected with ΔcolA or ΔcolA mutant or wild-type L. interrogans strain Lai for the indicated times. All urine specimens were centrifuged at 17,200 g for 15 minutes at 4°C to precipitate leptospiries. To count leptospiries in urine, the leptosprire-containing pellets from urine specimens collected on day 7 after challenge were suspended in phosphate-buffered saline and urine specimens at a 1:100 volume ratio, and the pellets from urine specimens collected on day 14 after the challenge were suspended in phosphate-buffered saline with the same volume of urine specimens. E, Counts of leptospiries of ΔcolA or ΔcolA mutant or wild-type L. interrogans strain Lai in urine specimens of hamsters for the indicated times. Bars show means ± SDs of colonies from 3 separate samples of ≥3 animals. *P < .05 (vs leptospiries in urine specimens from the hamsters infected with the ΔcolA mutant or wild-type strain).

Attenuated Virulence of ΔcolA Mutant in Hamsters

The 50% lethal dose in hamsters within 14 days after challenge was $2.4 \times 10^7$ leptospiries for the ΔcolA mutant, $1.0 \times 10^6$ leptospiries for the ΔcolA mutant, and $0.94 \times 10^6$ leptospiries for wild-type L. interrogans strain Lai. When $1 \times 10^6$ leptospiries were used as the challenge dose per animal, the wild-type strain or ΚΔcolA 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

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**Figure 6.** Histopathological damage in hamsters infected with *colA* gene–deleted (Δ*colA*) or *colA* gene–complemented (CΔ*colA*) mutant or wild-type *Leptospira interrogans* strain Lai. Lung, liver, and kidney specimens of hamsters were collected on day 7 after challenge with the Δ*colA* or CΔ*colA* 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 CΔ*colA* 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 Δ*colA* 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 pathological 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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**Potential conflicts of interest.** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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