Binding of Leptospira to Extracellular Matrix Proteins

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Background: *Leptospirosis is a zoonotic disease of global importance. Pathogenesis caused by this infectious disease remains unclear. Attachment of pathogenic leptospires to host tissues is a crucial initial step to establish the infection.*
Objective: *Study the binding of the spirochete to three types of extracellular matrix (ECM), collagen type IV, fibronectin, and laminin, which are major components of target organs.*
Material and Method: *ELISA-based experiments were performed to determine binding of pathogenic (serovar icterohaemorrhagie) and non-pathogenic (serovar Patoc) serovars, to purified ECM.*
Results: *Both pathogenic and non-pathogenic serovars bound to all three types of ECM in the dose-dependent manner and the binding to_fibronectin is higher than to collagen and laminin ($p < 0.005$).*
Conclusion: *Pathogenic leptospires can bind to various types of ECM and the binding of leptospires to fibronectin was higher than to collagen and laminin. However, this capability may not be the only mechanism that makes leptospires virulent since non-pathogenic leptospire can bind the ECM as well.*

Keywords: *Leptospirosis, Extracellular matrix, Collagen, Fibronectin, Laminin*

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Leptospirosis, which is caused by the spirochete *Leptospira interrogans*, is a zoonotic disease that is a public health concern worldwide. Pathogenesis of leptospirosis is not well understood. After leptospires move through skin or mucosal abrasions, the spirochete spreads hematogenously to multiple target organs resulting in systemic infection[1-6]. Binding of the organism to host tissues is an important step to establish the infection. The process requires the interaction between bacterial surface structures and host components[7-9]. The authors focused on the extacellular matrix (ECM) components located in the basement membrane of the endothelial or epithelial cell layer as a potential target for attachment. Damaged host tissues may expose the ECM and this allows microbial adherence and colonization. Studies on binding of leptospires to the ECM are limited. Three types of the ECM proteins, which are common components of the basement membrane, including the collagen type IV, fibronectin, and laminin, were used. The present study is aimed to determine the binding of pathogenic and saprophytic leptospires to purified ECM proteins of human origin.

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Material and Method

Bacterial strains and growth conditions

Leptospira interrogans serovar Icterohaemorrhagiae strain RGA and *L. biflexa* serovar Patoc strain Patoc I were kindly provided by AFRIMS. Both strains were grown in EMJH medium (Difco & BBL, Sparks, MD, USA) enriched with 10% albumin (Sigma, St Louis, MO, USA) at 30□C. The spirochetes were checked for contamination and then were subcultured every 5 to 7 days. Concentration of stationary-phase *Leptospira* was determined by counting with a Petroff-Hausser chamber and was adjusted to a concentration of 10^8/mL in phosphate-buffered saline (PBS) (138 mM NaCl, 2.7 mM KCl, 0.69 mM KH_2PO_4, 6.4 mM Na_2HPO_4 [pH 7.4]) before use. In some indicated experiments, serial dilutions of the leptospires were used.

Primary and secondary antibodies

Antisera specific to *L. interrogans* serovar Icteroheamorrhagie and *L. biflexa* serovar Patoc strain

Patoc I (KIT, Netherlands) were used as primary antibodies in the enzyme-linked immunuosorbent assay (ELISA)-based ECM binding assay. Secondary antibody used in the present study was peroxidase-conjugated goat anti-rabbit immunoglobulins (Igs; DAKO, Denmark).

Extracellular matrix (ECM) proteins

Purified collagen type IV (C-5533), plasma fibronectin (Fn, F-2006), and laminin (L-6274) of human origin, as well as the control protein, bovine serum albumin (BSA), were purchased from Sigma (St.Louis, MO, USA). All purified ECM proteins and BSA were prepared in carbonate-bicarbonate buffer (0.05 M $NaHCO_3$, 0.05 M Na_2CO_3, pH 9.6).

ECM protein binding assay

The ECM binding experiment was performed based on ELISA technique. Collagen, Fn, or laminin in PBS were coated on ELISA strip (Costar, Cambridge, MA, USA) at different concentrations at 4□C overnight. BSA at the same concentration was used as a control. The strips were washed three times with PBS and then blocked with blocking solution containing 3% gelatin for 1 h at 37□C. After removal of blocking solution, the strips were washed three times with PBS. One hundred microlitres of Leptospira at various concentrations was added to each well and then incubated for 3 h at 37□C. The same amount of PBS in the absence of leptospires was used as a control. Unbound leptospires were gently removed by washing with PBST (PBS containing 0.1% Tween 20) for three times. Serovar-specific antisera were added to the corresponding well at the dilution of 1:3,000 and the strip was incubated for 1 h at 37□C. After washing three times with PBST, a goat anti-rabbit Ig conjugated with peroxidase (DAKO, Denmark) at the dilution of 1:5,000 was used as a secondary antibody. The substrate for peroxidase, ophenylenediamine dihydrocholoride, was added and the strip was kept at room temperature in the dark. The reaction was stopped by adding 1 NH_2SO_4. The absorbance at 492 nm was read by a microplate reader (SmartSpec□3000, BIO-RAD, Hercules, CA, USA). Each experiment was performed in triplicate.

Results

Determination of optimal condition for ECM binding assay

An ELISA -based ECM protein-binding assay was performed to determine binding of leptospires to ECM. To determine the optimal amount of purified ECM proteins for the assay, collagen, Fn, and laminin at the concentrations of 10, 20 and 50 ug/mL were coated on the microtiter plate. In addition, different bacterial concentrations, incubation times, temperatures, types, and concentration of blocking agents, and various dilutions of primary and secondary antibodies were also tested.

Leptospires at the concentration of 10^4 to 10^8/mL and purified ECM at the concentration of 50 ug/mL gave the results in the optimal absorbance range that could be read by spectrophotometer and thus selected for further experiments. Other conditions used in the ECM protein-binding assay were as in the Material and Method.

Binding of leptospires to fibronectin, collagen, and laminin

The binding of pathogenic and non-pathogenic leptospires to different ECM proteins was determined using different concentrations of the spirochetes. Both pathogenic and non-pathogenic leptospires were able to bind all three types of purified ECM proteins including Fn, collagen, and laminin. The binding of both serovars to the purified ECM was shown to be increased with the number of organisms used (Fig. 1-3, respectively).

Leptospires bound to fibronectin more than to collagen and laminin

The binding of leptospires to three types of ECM proteins was compared by performing the assay at the same condition in the same experiment. Collagen, fibronectin, and laminin were coated on microtiter wells at the concentration of 50 ug/mL. The data demonstrated that *L. interrogans* serovar Icteroheamorrhagie and *L. biflexa* serovar Patoc strain Patoc I bound to all extracellular matrix proteins used. However, both pathogenic and non-pathogenic serovars bound to Fn more than to collagen and laminin (Fig. 4).

Discussion

Microbial adhesion is the initial critical event in the pathogenesis of most infections and the molecules involved in adhesion are promising targets for the development of new antimicrobial therapeutics. A class of cell surface adhesins that specifically interact with extracellular matrix components are designated MSCRAMMs (microbial surface components recognizing adhesive matrix molecules)[7-9]. MSCRAMMs have been studied as molecules involving in host tissue colonization and invasion, and as virulence

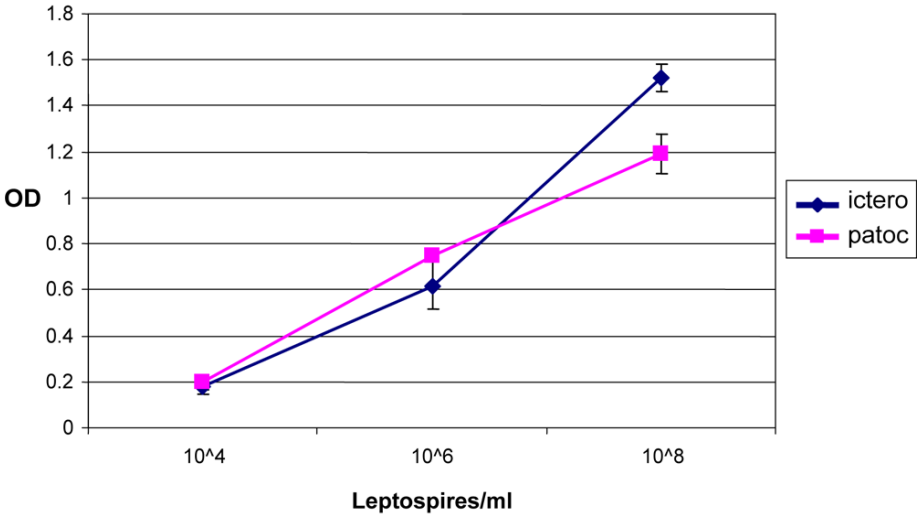

Fig. 1 Binding of leptospires to fibronectin (Binding of *L. interrogans* serovar Icteroheamorrhagiae and *L. biflexa* serovar Patoc strain Patoc I to fibronectin was determined by ELISA, fifty microgram/mL of fibronectin was coated onto microtiter wells, various concentrations of leptospires (10^4, 10^6, and 10^8 leptospires/mL) were added and ELISA was performed as described in Material and Method)

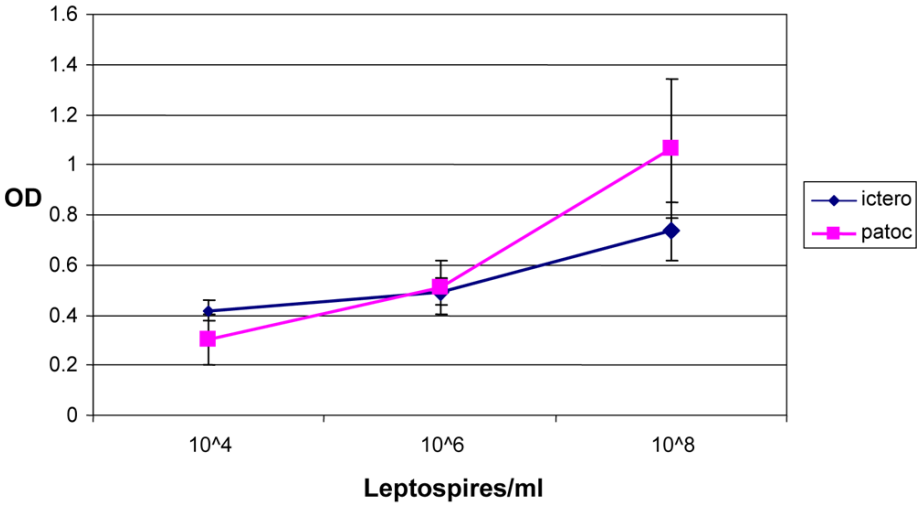

Fig. 2 Binding of leptospires to collagen (Binding of *L. interrogans* serovar Ictero heamorrhagiae and *L. biflexa* serovar Patoc strain Patoc I to collagen was determined by ELISA, fifty microgram/mL of collagen was coated onto microtiter wells, various concentrations of leptospires (10^4, 10^6, and 10^8 leptospires/mL) were added and ELISA was performed as described in Material and Method)

factors. The authors postulated that the ability of pathogenic *Leptospira* to bind to the extracellular matrix components of the basement membrane might be a mechanism that the spirochete uses to attach to host tissues prior to tissue invasion. The pathogenic *Leptospira* is able to disseminate from the skin or mucous membrane and infect several organs. Therefore, the authors chose to study three ECM proteins, fibronectin, collagen type IV, and laminin, that are ubiquitously found in the basement membrane. In

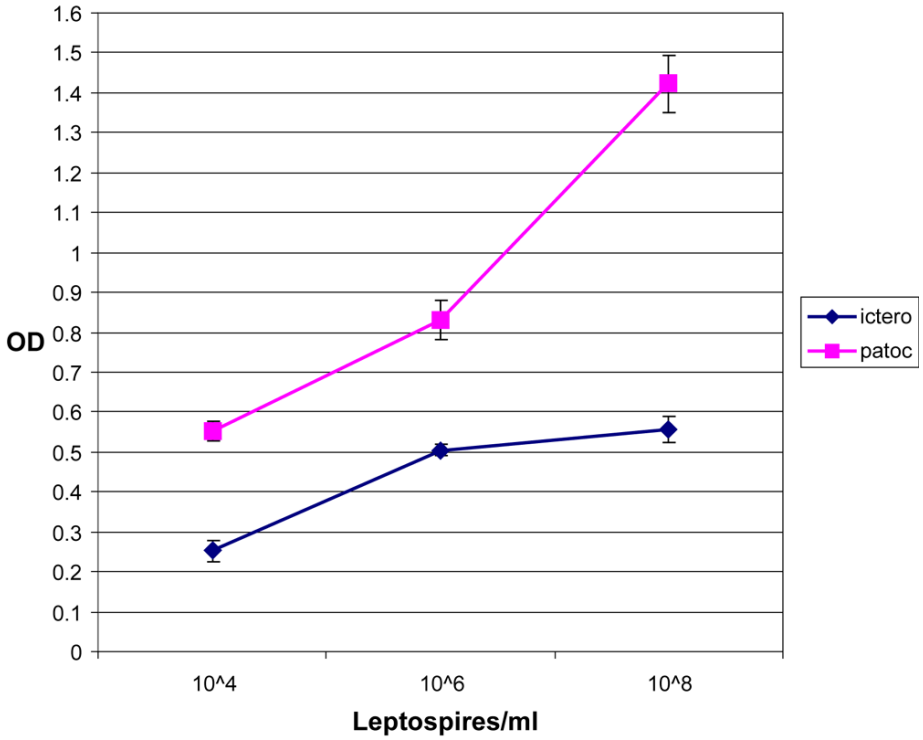

Fig. 3 Binding of leptospires to laminin (Binding of *L. interrogans* serovar Icteroheamorrhagiae and *L. biflexa* serovar Patoc strain Patoc I to laminin was determined by ELISA, fifty microgram/mL of laminin was coated onto microtiter wells, various concentrations of leptospires (10^4, 10^6, and 10^8 leptospires/mL) were added and ELISA was performed in the same way as done for binding to fibronectin and collagen)

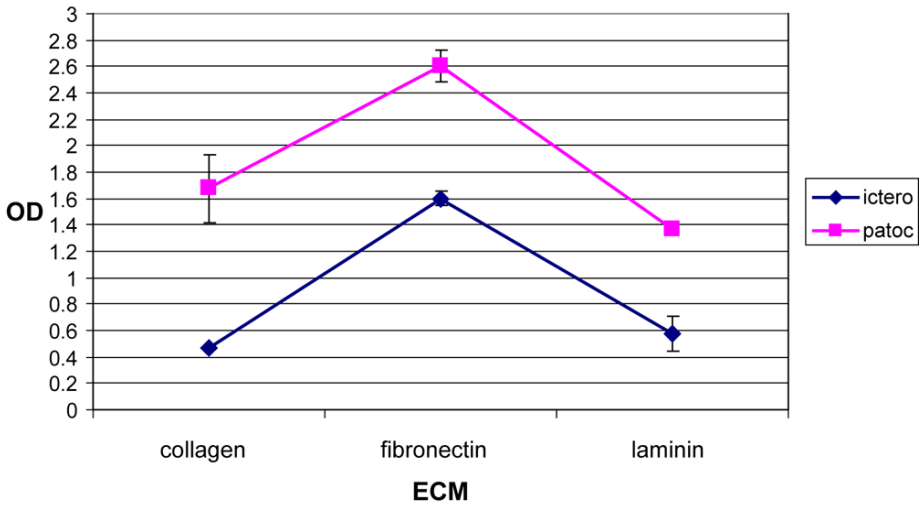

Fig. 4 Binding of leptospires to collagen, fibronectin, and laminin (Bindings of *L. interrogans* serovar Icteroheamorrhagiae and *L. biflexa* serovar Patoc strain Patoc I to fibronectin, collagen and laminin were performed as the same time, fifty microgram/mL of ECM and 10^8/mL of leptospires were used and ELISA was performed as done in previous experiments)

addition, they are common ECM proteins that have been previously reported as targets of host tissue binding in several organisms including spirochetes.

FnbpA of Staphylococcus aureus, Sfb of *Streptococcus pyogenes,* and FnbA and FnbB of *Streptococcus dysgalactiae,* were shown to bind to multiple sites in the N-terminal domain of Fn and this binding mediated adhesion to host tissue and bacterial uptake into non-phagocytic cells[8,10-14]. Besides binding to fibronectin, binding to collagen has been shown to be involved in pathology caused by *S. aureus*. The collagen adhesin of *S.aureus* (CNA) has been shown to be a virulence factor in several animal models. Isogenic strains of *S. aureus* expressing various forms of CNA have been compared in causing pathology in a mouse model of septic arthritis. It has been demonstrated that the virulence of CNA depends on its collagen-binding ability[15].

It has been suggested that binding of extracellular matrix proteins is a possible mechanism for *Salmonella enterica* serotype Typhimurium *(S. Typhimurium)* intestinal persistence. *S. Typhimurium,* expressed MisL, enable the organism to bind fibrone ctin to its cell surface, resulting in attachment to fibronectin and in increased invasiveness for human epithelial cells. In a mouse model of *S. Typhimurium* intestinal persistence, misL mutant was impaired in its ability to colonize the cecum[16]. The Hemophilus influenzae Hap autotransporter is an adhesin that promotes adherence to epithelial cells and extracellular matrix proteins such as fibronectin, laminin, and collagen IV and mediates bacterial aggregation and microcolony formation[17].

NcaA, collagen-binding protein of *Hemophilus ducreyi,* the etiologic agent of the sexually transmitted genital ulcer disease chancroid, is important for *H. ducreyi* in host colonization. Its role in pathogenesis has been shown in both swine and human models. The number of organisms isolated from lesions was less when animals or humans were challenged with ncaA mutant than with wide-type[18,19]. *Helicobacter pylori* has been shown to inhibit the interaction between the extracellular matrix protein laminin and its receptor on gastric epithelial cells, resulting in the loss of mucosal integrity. A 25-kDa outer membrane protein of *H. pylori* in association with the bacterial lipopolysaccharides (LPS) was shown to mediate the attachment to laminin[20]. Moreover, binding of *Treponema pallidum* and *Borrelia burgdorferi*, the spirochete bacteria, to ECM has been widely studied. These organisms colonize various tissues and their various MSCRAMMs have been identified even though their roles in pathogenesis are not clearly elucidated[21-31].

In the present study, the authors demonstrated that pathogenic *L. interrogans* serovar Icterohea-morrhagie bound to Fn, collagen type IV and laminin in a cell number-dependent manner. The authors were unable to quantitatively and accurately compare the binding capacity between pathogenic and non-pathogenic serovars since the binding assay was based on ELISA technique using unequal concentration of primary antibodies against each serovar. However, non-pathogenic serovar, *L. biflexa* serovar Patoc strain Patoc I, also bound to all three types of ECM protein tested in a similar manner to those of pathogenic serovar. Therefore, binding of pathogenic leptospires to these ECM proteins is not the only mechanism that renders them to be more virulent than the non-pathogenic serovar.

The binding capacity of each leptospiral serovar to different ECM proteins was compared by performing the binding assay on the same microtiter plate under the same condition. The binding of both leptospires to fibronectin was found to be higher than that to collagen type IV and laminin. A 36-kD fibronectin -binding protein was shown to be expressed on the cell surface of the virulent strain of *L. interrogans* serovar Icterohaemorrhagie but was not expressed in its isogenic avirulent variant and a saprophytic strain (*L. biflexa* serovar Patoc strain Patoc I).The present study showed that not only serovar Icterohae-morrhagie but also serovar Patoc was able to bind to fibronectin although the saprophytic serovar did not express the 36-kD fibronectin-binding protein. It is possible that the serovar Patoc will bind to fibronectin via other surface proteins. In the previous study, the interaction of the 36-kD fibronectin -binding protein with fibronectin was specific but was not involved the integrin-binding domain since RGDS synthetic peptides were not able to inhibit the binding of fibronectin[32]. The region of fibronectin that bound to this potential adhesin was found to overlap the gelatin-binding domain. Hence, the fibronectin-binding region of pathogenic serovar may be specific and distinct from that of non-pathogenic serovar. Another explanation is that the strain Patoc I used in our study is different from the strain used in the previous study. Surface leptospiral proteins that involve in other ECM binding have not been identified.

Conclusion

The present study shows that both patho-

genic and non-pathogenic serovars of *Leptospira* are able to bind to three major ECM constituents of the basement membrane including fibronectin, collagen type IV, and laminin. The binding of leptospires to fibronectin was higher than to collagen type IV and laminin. However, the binding to ECM may not be the only mechanism that makes leptospires virulent since non-pathogenic leptospire can bind the ECM as well. Leptospira serovar Patoc was previously shown that it did not express a 36-kD fibronectin-binding protein present in *Leptospira* serovar Icterohaemorrhagiae. Therefore, the saprophytic serovar may bind to fibronectin via a surface protein that is distinct from the pathogenic serovar.

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การจับของเชื้อเลปโตสไปรากับโปรตีนเมตริกซ์นอกเซลล์

จินตนา จิรถาวร, กนิษฐา ภัทรกุล, วุฒิไกร ศักดิ์สิทธิ์, ยง ภู่วรวรรณ

ภูมิหลัง: *เลปโตสไปโรซิสเป็นโรคติดต่อจากสัตว์สู่คนที่เป็นปัญหาทั่วโลก เชื้อก่อโรค คือ เชื้อแบคทีเรีย Leptospira interrogans ซึ่งพยาธิกำเนิดของโรคยังไม่ทราบแน่ชัด การจับของเชื้อกับเนื้อเยื่อของร่างกายเป็นขั้นตอนเริ่มต้นที่สำคัญในการก่อโรค*

วัตถุประสงค์: *เพื่อเปรียบเทียบการจับของเชื้อเลปโตสไปรากับโปรตีนเมตริกซ์นอกเซลล์ 3 ชนิด คือ collagen type IV, fibronectin และ laminin*

วัสดุและวิธีการ: *ศึกษาการจับของเชื้อกับโปรตีนเมตริกซ์นอกเซลล์โดยวิธี ELISA เชื้อที่ใช้ศึกษาได้แก่เชื้อชนิดก่อโรค คือ serovar icterohaemorrhagiae และชนิดไม่ก่อโรคคือ serovar patoc*

ผลการศึกษา: *เชื้อชนิดก่อโรคและชนิดไม่ก่อโรคสามารถจับกับ ECM ได้ทั้ง 3 ชนิด โดยเชื้อทั้งสองชนิดสามารถจับกับ fibronectin ได้ดีกว่า collagen และ laminin อย่างมีนัยสำคัญทางสถิติ ($p < 0.005$)*

สรุป: *เชื้อเลปโตสไปราชนิดก่อโรคและชนิดไม่ก่อโรคสามารถจับกับ ECM ได้ทั้ง 3 ชนิด ความสามารถในการก่อโรคของเชื้อไม่ได้เกิดจากการจับกับ ECM เท่านั้นเนื่องจากพบว่าเชื้อชนิดไม่ก่อโรคก็สามารถจับกับ ECM ได้*