Journal of Zhejiang University SCIENCE
ISSN 1009-3095
http://www.zju.edu.cn/jzus
E-mail: jzus@zju.edu.cn

Determination of the genus-specific antigens in outer membrane proteins from the strains of *Leptospira interrogans* and *Leptospira biflexa* with different virulence*

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Received May 29, 2003; revision accepted Aug. 25, 2003

Abstract: Objective: To determine the existence of genus-specific antigens in outer membrane proteins (OMPs) of *leptospira* with different virulence. Methods: Microscope agglutination test (MAT) was applied to detect the agglutination between commercial rabbit antiserum against leptospiral genus-specific TR/Patoc I antigen and 17 strains of *Leptospira interrongans* belonging to 15 serogroups and 2 strains of *Leptospira biflexa* belonging to 2 serogroups. The outer envelopes (OEs) of *L.interrogans* serogroup *Icterohaemorrhagiae* serovar *lai* strain *lai* (56601) with strong virulence and serogroup *Pomona* serovar *pomona* strain *Luo* (56608) with low virulence, and *L.biflexa* serogroup *Semaranga* serovar *patoc* strain *Patoc* I without virulence were prepared by using the method reported in Auran *et al.*(1972). OMPs in the OEs were obtained by treatment with sodium deoxycholate. SDS-PAGE and western blot were used for analyzing the features of the OMPs on electrophoretic pattern and the immunoreactivity to the antiserum against TR/Patoc I antigen, respectively. Results: All the tested strains belonging to different leptospiral serogroups agglutinated to the antiserum against leptospiral genus-specific TR/Patoc I antigen with agglutination titers ranging from 1:256–1:512. A similar SDS-PAGE pattern of the OMPs from the three strains of *leptospira* with different virulence was shown and the molecular weight of a major protein fragment in the OMPs was found to be approximately 60 KDa. A positive protein fragment with approximately 32 KDa confirmed by Western blot, was able to react with the antiserum against leptospiral genus-specific TR/Patoc I antigen, and was found in each the OMPs of the three stains of *leptospira*. Conclusion: There are genus-specific antigens on the surface of *L.interrogans* and *L.biflexa*. The OMP with molecular weight of 32 KDa may be one of the genus-specific protein antigens of *leptospira*.

Key words: *Leptospira*, Outer membrane protein, Genus-specific antigen
Document code: A **CLC number:** R377

INTRODUCTION

Infection by *L.interrogans* is characterized by hemorrhage, diarrhea, jaundice, severe renal impairment, and aseptic meningitis. Leptospirosis is spread from animal urine to humans and is considered to be the most widespread anthropozoonosis in the world. This disease is common in farmers and veterinarians, but can also be transmitted through contaminated water in flooded areas. In particular, leptospirosis is one of the most important infectious diseases contracted in waterlogging areas and rice paddies (Katz *et al.*, 2002; Kariv *et al.*,

* Project (No. 39970678) supported by the National Natural Science Foundation of China

2001; Kupek *et al.*, 2000). *Leptospira* is divided into *Leptospira interrogans* and *Leptospira biflexa.* The former is pathogenic for human and animals and the latter is saprophytic. So far, there are more than 230 different serovars belonging to 28 serogroups of *Leptospira interrogans* based on serological examinations (Levett, 2001). The currently used multivalent vaccine of *leptospira* in the world is made of dead whole cells of the microorganism (Lomar *et al.*, 2000). Since the cross immune protection among different serogroups of *L.interrogans* is weak or minimal, and there is large geographical variability among the dominant serovars (Guerreiro *et al.*, 2001; Kobayashi, 2001), one kind of vaccine is usually composed of 3–5 dominant serovars particular to the local species. Because of the limited cross-protection of the vaccine, anyone of *L.interrogans* serovars, which does not contain in the vaccine, might be highly possible to cause fulminant epidemic of leptospirosis (Sehgal *et al.*, 2000; Barcellos and Sabroza, 2001; Fuortes and Nettleman, 1994). Therefore, finding of genus-specific protein antigens of *L.interrogans* for further development of new vaccines is of great importance for control of leptospirosis. In previous studies, an antigen of *leptospira* named as TR/Patoc I antigen was shown to exist in all tested *L.interrogans* and *L.biflexa* serovars; and which could react to antibody in sera of patients infected with anyone of *L.interrogans* serovars through immune agglutination (Chapman *et al.*, 1990).

Significantly, antisera from animals immunized with the leptospiral TR/Patoc I antigen is also genus-specific, that is, could be agglutinated to almost of all the serovars of *L.interrogans* and *L.biflexa*. However, this genus-specific antigen itself has never been identified. And the biochemical characteristics of TR/Patoc I antigen and its cellular location on *leptospira* are unknown.

In this study, MAT by dark field microscopy was applied to detect the agglutination between the commercial rabbit antiserum against leptospiral genus-specific TR/Patoc I antigen and different strains of *L.interrongans* and *L.* bifrexa. By using SDS-PAGE and Western blot, the TR/Patoc I antigen was ascertained to be existing in the OMPs from strong and weakly virulent strains of *L.interrogans* and non-virulent strains of *L.bifrexa*. The results obtained will provide guidelines for further development of genus-specific vaccine of *leptospira*.

MATERIALS AND METHODS

Strains of *Leptospira* and cultivation

L.interrogan serogoup *Icterohaemorrhagiae* serovar *lai* strain *Lai* (56601) and serovar naam strain Naam (56620), serogroup *Javanica* serovar *javanica* strain *M* 10 (56602), serogroup *Canicola* serovar *canicola* strain *Lin* (56603), serogroup *Ballum* serovar *ballum* strain *Pishu* (56604), serogroup *Pyrogenes* serovar *pyrogenes* strain *Tian* (56605), serogroup *Autumnalis* serovar *autumnalis* strain *Lin* 4 (56606), serogroup *Australis* serovar *australis* strain 65–9 (56607), serogroup *Pomana* serovar *pomana* strain *Luo* (56608), serogroup *Grippotyphosa* serovar *lin* strain *Lin* 6 (56609), serogroup *Hebdomadis* serovar *hebdomadis* strain *P* 7 (56610), serogroup *Bataviae* serovar *paidjan* strain *L* 37 (56612), serogroup *Tarassovi* serovar *tarassovi* strain 55–52 (56613), serovar moldviae strain Dong 27 (56671), serogroup *Manhao* serovar *manhao* II strain *L* 105 (56615), serogroup *Sejroe* serovar *wolffi* strain *L* 183 (56635), serogroup *Mini* serovar *mini* strain *Nan* 10 (56655) and *L.biflexa* serogroup *Semaranga* serovar *patoc* strain *Patoc* I, serogroup *Andamana* serovar *andamana* strain *CH*11 were offered by the National Institute for the Control of Pharmaceutical and Biological Products of China (NICPBP). The 17 strains of *L.interrogan* are the major epidemic leptospiral serovars and used as the reference strains in China (Dai, 1992). EMJH medium was used to cultivate *leptospira* at 28 °C for 5–7 d. The fresh culture with 2×10^8 cells/ml of *leptospira* estimated by dark field microscopy is suitable for further usage.

MAT

The rabbit antiserum against leptospiral genus-specific TR/Patoc I antigen was offered by NICPBP. MAT through dark field microscope was performed as described in Cole *et al*. (1973). Briefly,

10 μl of the rabbit antiserum with different dilutions were mixed with 10 μl of the fresh culture of different strains of *leptospira* in 20-wells agglutination plates, respectively. The plates were shaken for 2 h at 37 °C. The agglutination states of the antibody and *leptospira* were examined by dark field microscopy. The MAT titer was the reciprocal of the highest dilution of serum in which ≥50% of the antigen was agglutinated (Cole *et al.*, 1973). At the same time, negative controls were prepared instead of the diluted antiserum with normal saline.

Preparation of leptospiral outer envelope

L.interrogan strain 56601 and *strain* 56608 and *L.biflexa strain Patoc* I were selected for preparation of outer envelope (OE). The former two are most prevailing in China and usually used as representatives of strongly virulent strains and weakly virulent strains, respectively. The latter is the most common internationally used strain of *L.biflexa*. The OEs from the three strains were prepared according to Auran's method (Auran *et al.*, 1972). In brief, after addition of 8% (W/V) NaCl at the final concentration, the culture of *leptospira* was incubated for 3 h at room temperature. The mixture was filtrated through 0.85–1.2 μm membrane and then the salted leptospiral cells were collected by using distilled water with 1/80 volume of the original culture. An equal volume of 0.04% (W/V) SDS solution was added to dissolve the OE and then the solution was centrifuged at 3000 r/min for 30 min. The supernatant containing the OE fraction was dialysed against 0.01 mol/L PBS (pH 7.2) at 4 °C for 48 h and then was filtrated through 0.45 μm membrane. The final obtained solution with slightly opalescent color contained the purified OE.

Preparation and quantity of OMP

The purified OE solution was supplemented with sodium deoxycholate to a final concentration of 0.5% (W/V), and incubated for 2 h at room temperature, then centrifuged at 44 000 r/min for 2 h to yield OMP. The pallete was dialysed against the same PBS mentioned above for 2–3 d. The protein concentration in the OMP preparation was determined by using Lowry's method (Sambrook *et al.*, 1989).

SDS-PAGE

SDS-PAGE using 10% (V/V) acrylamide separating gel was performed using the method of Laemmli (1970). The OMP samples were divided into the following three groups: (1) treated with 4 mol/L urea; (2) treated with 1% (V/V) β-mercaptoethand; (3) not treated. Electrophoresis was carried out for 5 h at 120 V and then the gel was stained by Coomassie blue R250.

Western blot

After the SDS-PAGE separation, the protein fragments in the gel were electro-transferred to nitrocellulose membrane (Millipore) under 100 V voltage at 4 °C for 1 h. Western blot was performed according to the method described by Sambrook *et al.*(1989). The rabbit antiserum against leptospiral genus-specific TR/Patoc I antigen at 1:500 dilution and the goat anti-rabbit HRP-labeled IgG serum (Jackson Immuno research) at 1:1000 dilution were used as the first and second antibody, respectively. The membrane was developed by ECL method according to the manufacturer's instructions.

RESULTS

Protein concentration of OMP preparations

The protein concentrations of OMP preparations from the three strains of *leptospira* were 0.711 mg/ml, 0.712 mg/ml and 0.710 mg/ml, respectively.

Results of MAT

All the tested strains of *leptospira* were able to combine with the antibody in the rabbit antiserum against leptospiral genus-specific TR/Patoc I antigen; at agglutination titers of 1:256–1:512 (Table 1). That indicated that TR/Patoc I antigen existed in each of the strains of *leptospira* and epitopes of the antigen located on the surface of the microorganism.

SDS-PAGE pattern

The SDS-PAGE patterns of the 4 mol/L urea

treated samples, the 1% (V/V) β-mercaptoethand treated samples, and non-treated OMP samples were similar, except for the slightly upward migration of the bands from the β-mercaptoethand treated OMP samples compared with the other two. Despite the difference of the origins and treatment methods of the OMP preparations, a strongly stained band was observed at the position of approximately 60 KDa. This 60 KDa protein made up 70% or more of the total proteins in OMPs (Fig.1).

Result of Western blot

All the bands which could be recognized by TR/Patoc I antiserum were distributed in the region of molecular weight less than 43 KDa. The migration patterns of the OMPs from *L.interrogans* strain 56601 and 56608 were similar to each other, but different from those of *L.biflexa* strain *Patoc* I. Significantly, an obvious positive protein band with approximately 32 KDa was found in all the 3 tested strains of *leptospira* (Fig.2).

DISCUSSION

Since genus-specific protein antigens of *Leptospira* show high potential for development of novel universal vaccines and detection methods for further control of leptospirosis in human and animals (Guerreiro *et al.*, 2001), investigations for finding leptospiral genus-specific protein antigens are being actively conducted. As mentioned in the introduction, the extensive cross immunoreaction of the leptospiral TR/Patoc I antigen and the sera of patients suffering from leptospirosis strongly indicated the existence of genus-specific antigens of *leptospira*. Haake and his colleagues found two genes existed in the 5 strains belonging to different serovars of *L.interrogans* (Haake *et al.*, 2000). One of them was named as *OmpL*1 encoding a putative 38 KDa peptide with 320 amino acid residuals and another was named as *LipL*32 encoding a putative 32 KDa peptide with 267 amino acid residuals. Up to date, no definite evidences, however, for the exi-

Table 1 MAT results of the leptospial genus-specific TR/Patoc Ⅰ antiserum and the 19 strains of *leptospira*

Strain	MAT titer	Strain	MAT titer	Strain	MAT titer
56601	1:256	56608	1:256	56635	1:256
56602	1:512	56609	1:256	56655	1:256
56603	1:256	56610	1:256	56671	1:256
56604	1:256	56612	1:256	*Patoc* I	1:512
56605	1:256	56613	1:256	*CH*11	1:512
56606	1:512	56615	1:256		
56607	1:256	56620	1:256		

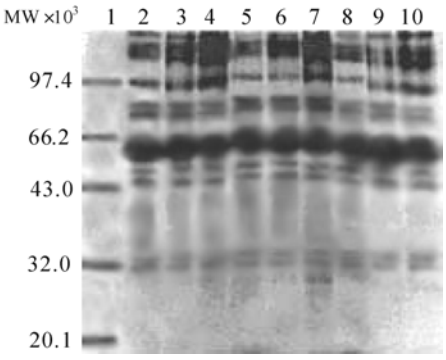

Fig.1 SDS-PAGE pattern of the OMPs from three strains of *leptospira* with different virulence

1: Molecular weight marker; 2−4: the OMPs from strain Patoc I, 56601 and 56608 treated with 4 mol/L urea; 5−7: the OMPs from strain Patoc I, 56601 and 56608 treated with 1% (V/V) β-mercaptoethanol; 8−10: the OMPs from strain Patoc I, 56601 and 56608 without any treatments

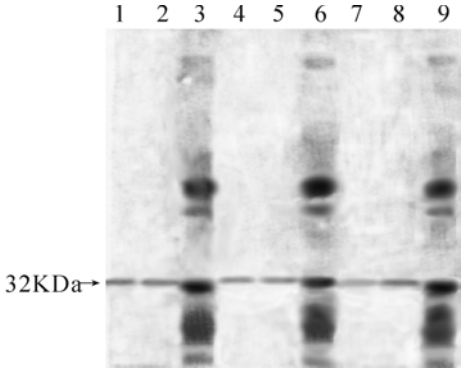

Fig.2 Western blot result of the TR/Patoc I antiserum and the OMPs of the 3 strains of *leptospira* with different virulence

1−3: the OMPs from strain 56608, 56601 and Patoc I without any treatments; 4−6: OMPs from strain 56608, 56601 and patoc Ⅰ treated with 1% (V/V) β-mercaptoethanol; 7−9: the OMPs from strain 56608, 56601 and Patoc I treated with 4 mol/L urea

stence of leptospiral genus-specific protein antigens have been obtained.

Leptospiral genus-specific TR/Patoc I antigen put on a pallet of fresh culture of *L.biflexa* serovar *patoc* strain *Patoc* I was centrifuged at 10 000 r/min and then heated at 80 ºC for 10 min (Chapman *et al.*, 1990). This procedure yielded a dead whole cell antigen of *leptospira*. Our finding revealed that this TR/Patoc I antiserum could react with the 17 strains of *L.interrongans* belonging to 15 serogroups and the 2 strains of *L.biflexa* belonging to 2 serogroups, indicating this genus-specific antigen generally existed in different serogroups of *leptospira*. Since living *leptospira* was used in MAT, the results mentioned above suggested that the TR/Patoc I antigen was located on the surface of *leptospira*.

Similar SDS-PAGE results indicated that OMPs from different leptospiral strains and by treatment methods used in this study did not affect their migration patterns. It is well known Coomassie blue R250 routinely used in SDS-PAGE is a stain specially used for protein staining. Western blot results showed that the strongly stained major 60 KDa protein bands from the three strains of *leptospira* in SDS-PAGE did not react on the antiserum against the *leptospira* genus-specific TR/Patoc I antigen. Significantly, the 32 KDa protein bands from all the OMP preparations of the three strains showed in SDS-PAGE that then could obviously and exactly combine to the antiserum, indicating this protein is a genus-specific antigen of *leptospira*. The much similar molecular weights of the 32 KDa protein and the putative *LipL*32 protein implied possible close correlation of the two proteins. However, many of questions about the leptospiral genus-specific 32 KDa protein antigen such as its biochemical and immunological characterristics remain to be further studied.

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