Contribution of Fucose-Containing Capsules in *Klebsiella pneumoniae* to Bacterial Virulence in Mice

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Bacterium *Klebsiella pneumoniae* (KP) contains a prominent capsule. Clinical infections usually are associated with pneumonia or urinary tract infection (UTI). Emerging evidence implicates KP in severe liver abscess especially in diabetic patients. The goal of this study was to investigate the capsular polysaccharides from KP of liver abscess (hepatic-KP) and of UTI-KP. The composition of capsular polysaccharides was analyzed by capillary high-performance liquid chromatography (HPLC, Dionex system). The terminal sugars were assayed by binding ability to lectins. The results showed that the capsule of a hepatic KP (KpL1) from a diabetic patient contained fucose, while the capsule from UTI-KP (KpU1) did not. The absence of fucose was verified by the absence of detectable polymerase chain reaction (PCR) fragment for fucose synthesis genes, *gmd* and *wcaG* in KpU1. Mice infected with the KpL1 showed high fatality, whereas those infected with the KpU1 showed high survival rate. The KpL1 capsule was reactive to lectins *AAA* and *AAL*, which detect fucose, while the KpU1 capsule was reactive to lectin *GNA*, which detects mannose. Phagocytosis experiment in mouse peritoneal cavity indicated that the peritoneal macrophages could interact with KpU1, while rare association of KpL1 with macrophages was observed. This study revealed that different polysaccharides were displayed on the bacterial capsules of virulent KpL1 as compared with the less virulent KpU1. Interaction of KpU1 with mice peritoneal macrophages was more prominent than that of KpL1. The possession of fucose might contribute to KpL1 virulence by avoiding phagocytosis since fucose on bacteria had been implicated in immune evasion. Exp Biol Med 233:64–70, 2008

Key words: bacterial capsule; diabetes mellitus; enzyme-linked immunosorbent assay; fucose; lectin binding assay; liver abscess

Introduction

Besides more common related diseases, such as mild urinary tract infections (UTIs) or pneumonia (1), infections with *Klebsiella pneumoniae* (KP) frequently cause severe symptoms as attested in several recent reports (2–4). Infections can be acquired in hospital or in community (5). Bacterium KP is a member of *Enterobacteriaceae* whose presence is ubiquitous because it is part of the normal flora in human gastrointestinal tract. The latter serves as a reservoir and is often the latent source for opportunist infections. Emerging evidence in Asia indicates that KP causes severe liver abscess, especially in diabetic patients (6, 7). Drug-resistant infectious KP strains have been isolated worldwide (8–12).

A prominent characteristic of KP is its mucoid colony appearance. Bacterial capsule is a viscous layer that consists of high molecular weight polysaccharides. In capsulated bacteria, the capsules are the bacterial components that are in contact with the host immune system. Induction of protective immunity with KP capsule has been shown to prevent fatal burn wound sepsis in mice (13). Possession of capsule in bacteria is considered an important virulence factor (14, 15), as the bacterial capsule may evade phagocytosis and impede bacterial clearance from the host.

Macrophages are known to recognize bacterial surface with mannose. Keisari *et al.* (16) reported that macrophages

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can recognize KP with mannose capsule. KP with loose, thin capsule is reported to be avirulent (17). Rapidly cleared KP strain k21a was shown to be less virulent, and k2 strain that is viable in blood is highly virulent (18). The binding of k21a to macrophage is responsible for its clearance, and the binding is inhibited by yeast mannan. The KP k2 capsular polysaccharide has been reported to contain glucose, mannose, and N-acetyl-glucuronic acid. (19).

Despite the prevalence of KP infections in liver and urinary tract, the UTI-KPs seldom spread to liver, suggesting that different virulence factors may be involved in these two types of KP infections. Besides the thickness of the bacterial capsules, the heterogeneity of capsular composition also constitutes the differences in KP virulence. The reason that for the recent emergence of virulent KP strains that can cause severe liver abscess in diabetic patients is unknown. We hypothesize that the capsules from virulent KP may contain molecular moiety that is related to the bacterial virulence.

Materials and Methods

Bacterial Strains. KpL1 was a KP strain isolated from a diabetic patient with liver abscess (hepatic KP). This strain displayed high virulence in diabetic and nondiabetic mice (20). KpU1 was a KP strain isolated from a nondiabetic patient with UTI. This strain had been shown to be less virulent in diabetic mice and nonvirulent in nondiabetic mice. Another hepatic strain, KpL2, and two other UTI strains, KpU2 and KpU3, were also obtained for the study. The biochemical profiles of these KP strains were checked with API20E strip (BioMerieux Inc., Hazelwood, MO) and all had typical profile of KP.

Serotyping of the Bacterial Strains. *Klebsiella* antisera "Seiken" (Denka Seiken Co., Ltd., Tokyo, Japan) was used for KP serotypings. The kit contained antibodies against KP capsular K1 to K6 serotypes. A colony of bacteria cultured on blood agar plate was suspended in 0.9% NaCl. A drop of antiserum was mixed with 10-µl bacterial suspension, and the reaction was rocked back and forth gently for 1–2 mins. Agglutination was interpreted as positive reaction. A control without antiserum was used as negative test.

Bacterial Capsule Purification. As the KP capsule was reported to be heat stable (21, 22), we modified the capsule preparation accordingly. The bacterial capsular polysaccharides (cps) were purified according to method described (13) with modification. Briefly, each bacterium was cultured in five Luria-Bertani (LB) agar plates overnight, and the bacteria were collected with a cotton swab and suspended in phosphate buffered saline (PBS, 137m*M* NaCl, 20m*M* KCl, 82m*M* Na_2HPO_4, 1.47m*M* KH_2PO_4; pH 7.4). The bacterial suspension was heated at 70°C for 30 mins, and a loopful of bacteria was subcultured to LB agar plate to ensure no survival. The suspension was moderately shaken, centrifuged at 13,000 *g* for 1 hr at 4°C, and the supernatant was collected. Ten percent Cetavlon was added to the supernatant to a final concentration of 0.5%, and the mixture was stirred at room temperature overnight. Precipitate was collected by centrifuging at 10,000 *g* for 30 mins and dissolved in 1 *M* $CaCl_2$. Ethanol was then added to a final concentration of 80% to precipitate the cps. The precipitate was dissolved in distilled water. The cps solution was then extracted three times with equal volume of chloroform-butanol (5:1); the water phases were pooled and dialyzed against distilled water. The dialysate was centrifuged 100,000 *g* for 16 hrs to remove lipopolysaccharides (LPS). The supernatant was precipitated again with 80% ethanol, and the pellet was dissolved in water. The cps solution was then treated with polymyxin B agarose (Sigma-Aldrich Co., St. Louis, MO) before assay.

Bacterial Capsular Composition Analysis by High-Performance Liquid Chromatography (HPLC). For neutral sugar analysis, purified capsule was treated with 1.56*N* trifluoroacetic acid at 100°C for 4 hrs. The reaction mixtures were dried in a vacuum and subsequently reconstituted in distilled water. The hydrolysates were used for analysis on HPLC (Dionex system, Dionex Corp., Sunnyvale, CA). The column used for HPLC analysis was Carbo Pac PA1 (Dionex); the sample volume injected was 50 µl (about 16.7 µg); and the eluent 16m*M* NaOH was used for neutral sugar at flow rate of 1 ml/min. Pulse amperometric detector (PAD) was used for detection with the signal of nanoCoulomb (nC). This assay was performed at Glyconex Inc. (Taiwan).

Lectin Binding Assay. Lectins were purchased from Sigma (St. Louis, MO) and biotinylation of lectins followed the method of Duk *et al*. (23). For binding assays, purified capsules at concentration of 200 ng per well in 50 µl coating buffer (0.05*M* carbonate buffer, pH 9.6) was coated on a 96-well MaxiSorp plate (Nalge NUNC International, Naperville, IL) at 4°C overnight. The plate was washed three times for 1 min each with Tris-buffered saline (TBS)-Tween (0.05*M* Tris-HCl, 0.15*M* NaCl, 0.05% Tween-20) and blocked with blocking reagent (0.5% bovine serum albumin in coating buffer) for 1 hr at room temperature with mild shaking. The plate was washed again as before and reacted with 5–20 ng biotinylated lectins per 50 µl TBS (0.05*M* Tris-HCl, 0.15*M* NaCl; pH 7.3) per well at room temperature for 1 hr with mild shaking. The plate was washed as before and 50 µl ExtrAvidin conjugated with alkaline phosphatase (Sigma-Aldrich) at 1:10,000 dilution in TBS was added. The plate was incubated at room temperature for 1 hr with mild shaking, washed as before, and rinsed with 1:100 dilution of substrate buffer (coating buffer containing 1m*M* $MgCl_2$) before adding 50 µl substrate that comprised 1 tablet of Sigma 104 (p-nitrophenyl phosphate) dissolved in 5 ml substrate buffer for color development. The color developed was read at 405 nm on an enzyme-linked immunosorbent assay (ELISA) reader (OP SYS MR, Thermo Labsystems, Chantilly, VA).

For enzyme treatment, each µg of KpU1 cps was

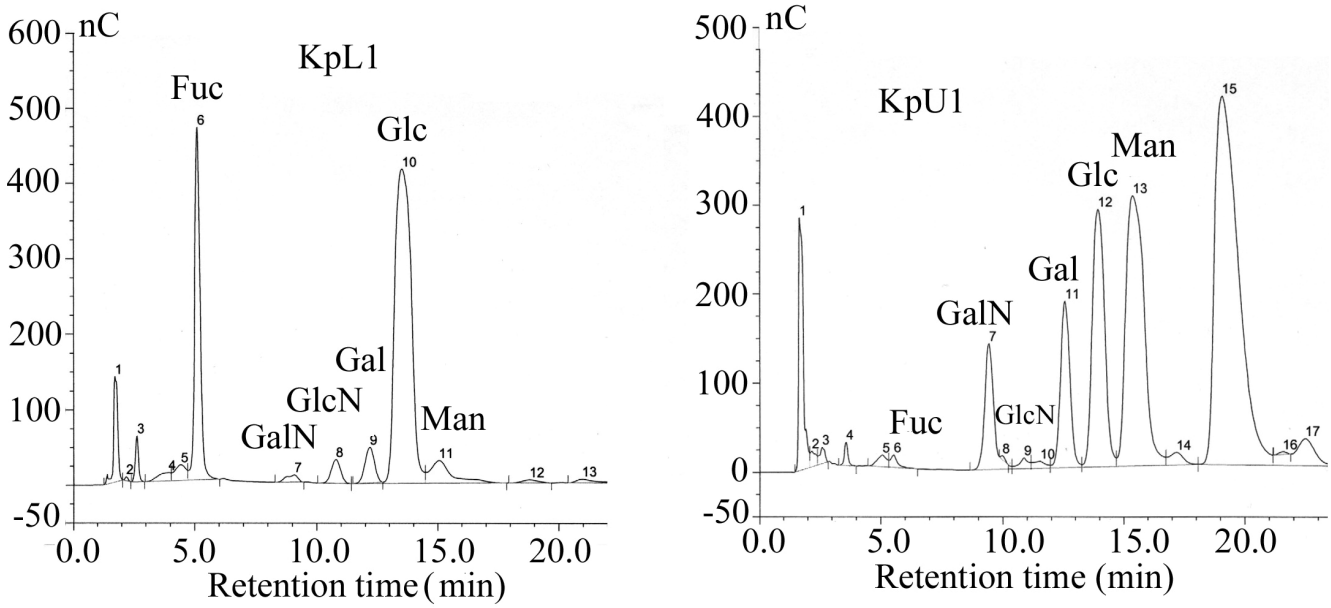

Figure 1. The HPLC profile of capsular polysaccharides. Fuc, fucose; GalN, N-acetylgalactoseamine; GlcN, N-acetylglucoseamine; Gal, galactose; Glc, glucose; Man, mannose.

treated with 5 mU α-mannosidase (Boehringer Mannheim GmbH, Germany) or 10 mU β-mannosidase (Sigma) and incubated at room temperature for 4 hrs. Each μg of KpL1 cps was treated with 2 mU neuraminidase (from *Clostridium perfringens*, Boehringer Mannheim) at 37°C for 2 hrs and 0.2 mU fucosidase (kindly supplied by C. H. Lin, Academia Sinica, Taiwan) at 37°C for 4 hrs. The treated and untreated samples were tested for lectin binding activity by ELISA on the same 96-well plate. The results represented the average of four experiments. Comparisons between treated and untreated results used SigmaPlot (SYSTAT Software Inc., San Jose, CA) *t* test.

PCR for Fucose Synthesis Genes of *K. pneumoniae*. PCR for KP GDP-mannose 4,6–dehydratase (*gmd*) used 5′ GATGTCCCACCTAATTCTGTAGTAG 3′ as sense primer and 5′ AACCATACCATTATACC CAGCAACA 3′ as antisense primer. For KP GDP-keto-6-deoxymannose 3,5 epimerase/4-reductase (*wcaG*) gene, 5′ GGTAAGAAAGGATCTTGAGGCAGCT 3′ as sense primer and 5′ TCAATTGAGATTAGCGGCGTATTCT 3′ as antisense primer were used. Both *gmd* and *wcaG* genes were required in bacteria to convert mannose to fucose. The amplified PCR fragment for *gmd* was 1262 base pair (bp) and for *wcaG* was 1117 bp.

Animal Experiments. All animal experiments followed the guidelines outlined in the Handbook of Laboratory Animal Care of the National Laboratory Animal Breeding and Research Center, National Science Council of Taiwan and were approved by the Animal Committee in Chang Gung University. Only male ICR mice (*Mus musculus* CD-1) of 6 to 8 weeks old were used. The mice were supplied by and housed at the Animal Center of Chang Gung University. Mice were kept in a temperature of 21–23°C with a relative humidity of 50%–70% and a 12:12-hr light:dark cycle with normal chow feeding.

Mice were injected by intraperitoneal (ip) route with 5 $\times 10^5$ colony forming unit (cfu) of KP suspended in saline. The mice were subsequently observed for 15 days for survival. For interaction with phagocytes, the mice were treated with thioglycollate for 72 hrs and ip-injected with 5 $\times 10^5$ cfu of KP. The peritoneal macrophages were harvested after 10 mins, spread on a sterile Petri dish, and stained with Giemsa stain (Sigma). Oil immersion microscopy was used to observe the interaction of bacteria with macrophages.

Results

Serotyping of KP Strains. Except for KpL2, which showed strong agglutination as *Klebsiella* K1 serotype, the KpL1, KpU1, KpU2, and KpU3 did not show agglutination.

Different Sugar Composition in Capsules of Hepatic *K. pneumoniae* and UTI Strains. Capillary HPLC analysis revealed prominent fucose and glucose peaks in the capsule of hepatic-KP (KpL1) isolated from a diabetic patient with liver abscess. The capsule of UTI-KP (KpU1) displayed prominent mannose, but no fucose (Fig. 1). Another hepatic strain KpL2 and two other UTI strains (KpU2 and KpU3) were obtained for capsular polysaccharide composition analysis, and the results were similar to KpL1 and KpU1, respectively (data not shown).

KpL1 Contained Complex Termini, While KpU1 Displayed Mannose at Capsule Surface. Lectin *Anguilla anguilla* agglutinin *(AAA)* that detects fucose (*Fuc*) 1→2 linkage (24), *Aleuria aurantia* lectin *(AAL)* that detects *Fuc* 1→6 or 1→3 linkage, as well as lectin *Artocarpus integrifolia* agglutinin *(AIA)* that detects *Gal*

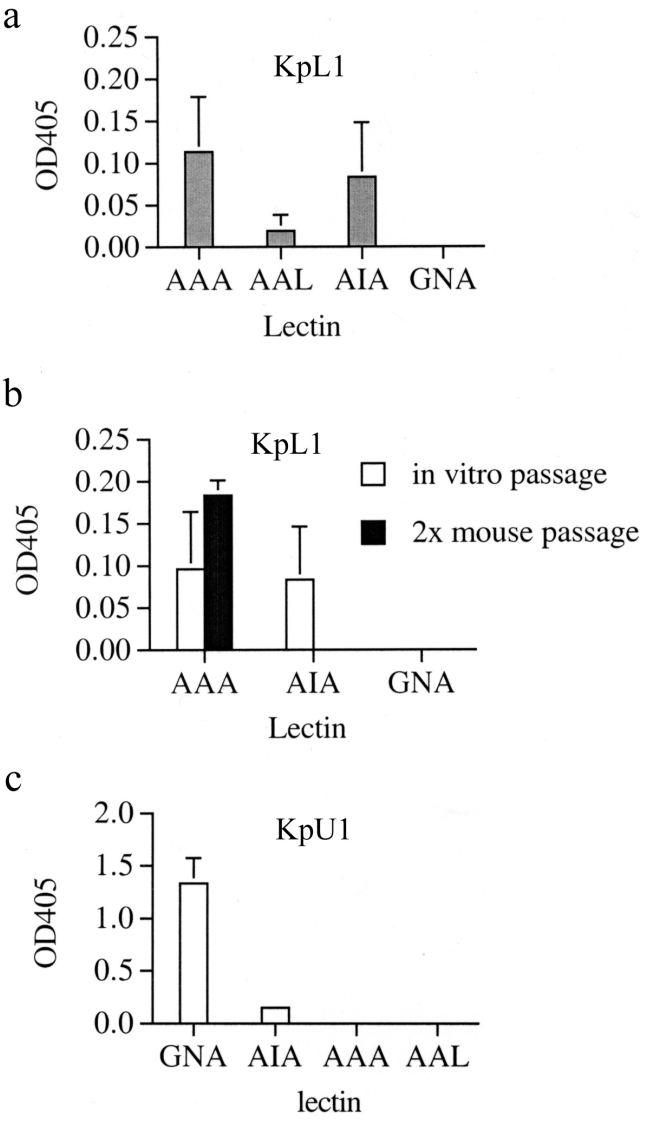

Figure 2. Binding profile of KP cps (200 ng) to various lectins by ELISA. (a) KpL1 after several months *in vitro* passages. (b) KpL1 after *in vitro* passages and after 2× mouse passages. (c) KpU1. The figure depicts the mean of four experiments with the standard error of the mean. Lectins *AAA* and *AAL* detect fucose; *AIA* lectin detects galactose; lectin *GNA* detects mannose.

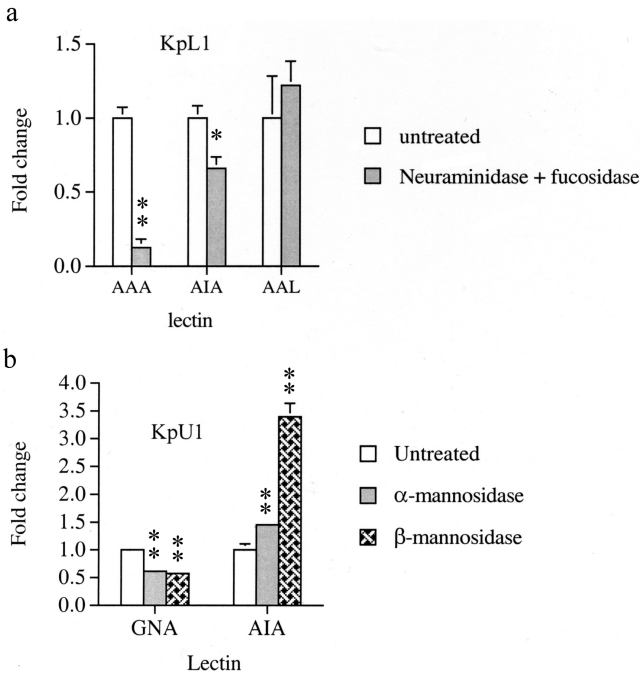

Figure 3. Effect of enzyme treatment to the binding profile of KP cps to lectins. (a) KpL1 before and after treatment with neuraminidase and fucosidase. (b) KpU1 before and after treatment with α-mannosidase or β-mannosidase. Columns indicate fold changes relative to the activity of respective untreated samples. The figure represents an average of four experiments with standard error of the mean. The * denotes $P < 0.05$ and ** denotes $P < 0.01$ for comparisons between the treated and respective untreated values.

were reactive to KpL1 capsule. Reactivity to lectin *Galanthus nivalis agglutinin* (*GNA* for mannose) was not apparent (Fig. 2a). The reactivity of *AAA* and *GNA* was also examined in KpL1 capsule isolated after passage two times in mice, and the results indicated that only *AAA* reactive sugar group was detected (Fig. 2b). Purified KpU1 cps were examined by lectin binding assays for terminal sugars. The results indicated that KpU1 cps reacted with biotinylated *GNA* and *AIA* but not with lectin *AAA* or *AAL* (Fig. 2c).

Treatment of KpL1 with neuraminidase and fucosidase reduced *AAA* reactive sugar group (Fig. 3a). The results indicated that KpL1 contained terminal sialic acid and fucose α1→2 linkage that is accessible by fucosidase after removal of sialic acid. Treatment of KpU1 capsules with mannosidase reduced the amount of mannose (*Man*) reactive to *GNA* lectin, at the same time *AIA*-reactive sugar group *Gal* became the major reactive sugar (Fig. 3b). The results suggested that the KpU1 capsules contained the sugar group *Man* attached to *Gal*.

KpU1 Interacted with Mouse Peritoneal Macrophages, While KpL1 Did Not. To examine whether the KpU1 interacts with macrophages in mouse peritoneal cavity, the phagocytes were induced in mouse peritoneal cavity with thioglycolate solution for 72 hrs before bacterial infection via ip. The alignment of KpU1 bacteria around macrophages was clearly seen (Fig. 4a), whereas the interaction of KpL1 with mouse peritoneal macrophages was rare (Fig. 4b). The KpL1 could invade liver tissue in our previous studies (20), but this phenomenon did not occur in mice with KpU1 infection. The results suggest that KpL1 might not be readily picked up by macrophages. The impaired clearance might permit the bacteria to multiply in blood to high concentration and invade liver tissues. The phenomenon also corresponded to the high fatality rate displayed by KpL1 as compared with KpU1 in mice ip-infected with 5×10^5 cfu/ml bacteria (Fig. 5).

Fucose Synthesis Genes *gmd* and *wcaG* Are in Most Hepatic *K. pneumoniae* But Not in UTI Strains. In order to verify our observation of the capillary HPLC analysis, fucose synthesis genes of KP, *gmd* and *wcaG*, were examined in hepatic and UTI strains. Total

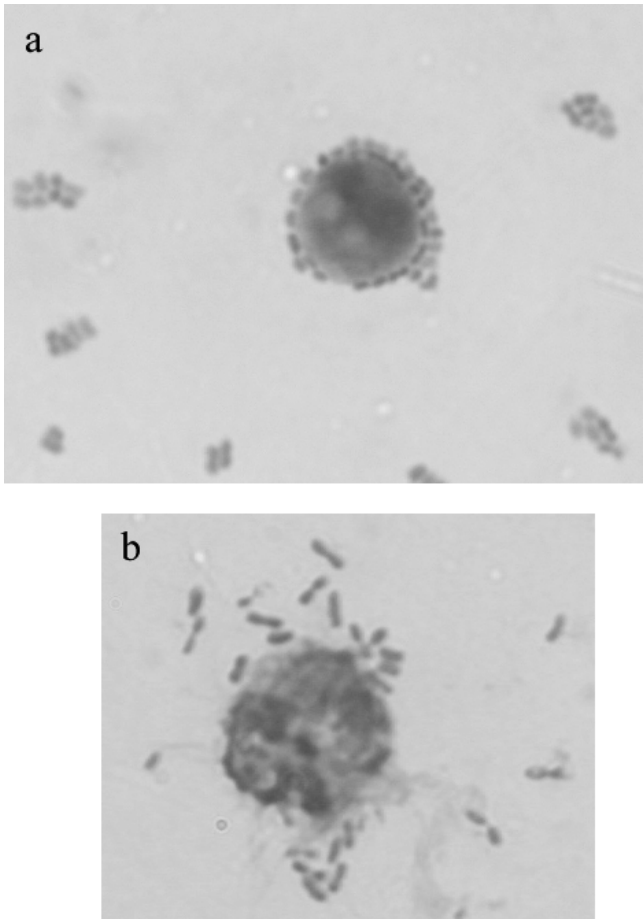

Figure 4. Interaction of KpU1 (a) and KpL1 (b) with mouse peritoneal macrophages. The bacteria can be seen to align on the periphery of a macrophage on (a), but not on (b).

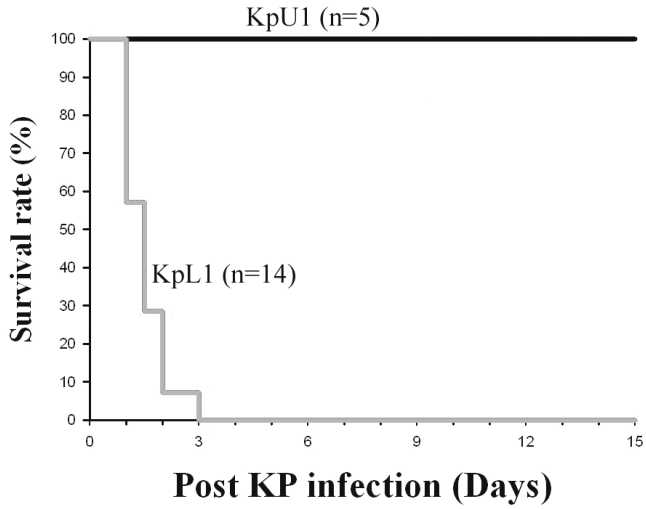

Figure 5. The survival curve of mice after ip injection of 5×10^5 cfu of KpL1 ($n = 14$) and KpU1 ($n = 5$) bacteria.

bacterial genomic DNA was isolated by cetyltrimethyl ammonium bromide (CTAB)/NaCl procedure (25) and primers for the two genes involved in the transformation of mannose to fucose were used for amplification by PCR. Our results indicated that the two hepatic KP strains, KpL1 and KpL2, all contained the correct DNA fragment for *gmd* and *wcaG* genes, and these DNA fragments were verified to be the correct genes by DNA sequence analysis. The three UTI-KP strains, however, did not yield the correct amplified fragments (Fig. 6). Further examination of clinical isolates of hepatic-KP strains revealed that 72% (18/25) hepatic-KP contained the fucose synthesis genes, but none (0/8) in the UTI-KP. The results indicated the fucose synthesis genes resided in the majority of liver abscess KP strains.

Discussion

K. pneumoniae contain very heterogeneous capsular epitopes. More than 80 capsular serotypes have been reported (26). Our results for serotyping showed that KpL2 was serotype K1, whereas other KP strains including KpL1, KpU1, KpU2, and KpU3 were not detected as serotype K1 to K6. Besides serotypings, differences in genetic composition were also characterized by using both random amplified polymorphic DNA (RAPD) and pulse-field gel electrophoresis (PFGE). The results showed that all five strains were genetically distinct (data not shown).

The KP that displayed serotype K1 capsule was reported to possess *magA* gene (27). MagA is an outer membrane protein with a molecular weight of 43 kd (28). On the KP capsule operon, the *magA* gene is located very close to *gmd*/*wcaG* genes. In both hepatic-KP strains, the KpL1 and KpL2, all showed presence of *gmd*/*wcaG* genes as well as *magA* by PCR experiment. Although our KpL1 possessed the *magA* genes, it did not yield visible agglutination with K1 antiserum. The possible reason is that the MagA responsible for K1 capsular epitope may be masked by some other sugar groups or modification that rendered K1 epitope negative.

Fucose is a sugar that is frequently used by bacteria for adaptation to survive within host environment. *Bacteroides* displays numerous surface capsular polysaccharides and glycoproteins with L-fucose (29). It gives the bacteria advantage in colonizing mammalian intestine under competitive conditions and renders them immunologically inert. Fucose containing lipopolysaccharide (LPS) in *Helicobacter pylori* serves as molecular mimicry between the host and the pathogen possibly for immune evasion (30). The possibility that KPs use the same strategy and survive in host cannot be ruled out.

Digestion of the KpL1 cps with neuraminidase and fucosidase reduced lectin *AAA* binding activity, indicating that fucose might be linked to the capsular sugar *Gal* residue by $\alpha 1 \rightarrow 2$ linkage. Our ELISA experiment indicated that the KpU1 expressed mannose and could interact with mouse peritoneal macrophages. This finding corresponded to our previous observation in which intravenous injection of KpU1 did not cause any deaths in nondiabetic mice (20). On the contrary, KpL1 could not interact with mouse peritoneal

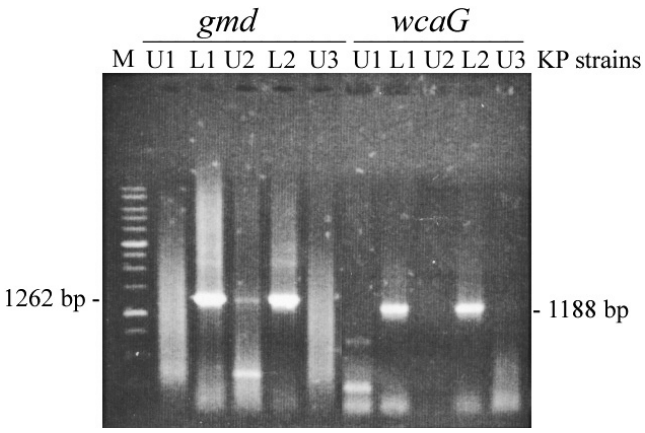

Figure 6. Amplification of *gmd* and *wcaG* genes for KpL1 (L1), KpL2 (L2), KpU1 (U1), KpU2 (U2), and KpU3 (U3) by PCR. Target band for *gmd* is 1262 bp and for *wcaG* is 1188 bp. Lane M, DNA marker.

macrophages and caused a high fatality rate in mice infected intravenously. This phenomenon was also observed when we infected the KP bacteria by ip injection (this study). This indicated that the KpL1 had the ability to invade and survive in the host system without being cleared by macrophages and that the possession of fucose in KpL1 capsule could potentially contribute to this immune evasion.

In the composition analysis, we observed that the KP strains isolated from the diabetic patients with liver abscess (the KpL1) expressed prominent glucose and fucose, indicating KP might preferentially use the available sugar in blood to incorporate into its capsule. Of interest, *in vivo* passage through mice rendered the capsule only reactive to lectin *AAA* in our ELISA experiment. It seemed that KP growing in different conditions for a period of time, such as in culture media or in a mouse host, displayed different surface epitope on capsules. Environmental conditions might play an important role in KP capsular surface epitopes. This finding is not unique in the microbial world. The fucose-containing Lewis antigen expression in *H. pylori* was reported to be variable depending on the host Lewis antigen categories (30). Expression of fucose in *in vivo* condition could contribute to KP survival in host.

The fact that more than 70% of clinical isolates from liver abscess patients displayed presence of fucose synthesis genes and none of the UTI-KP strains possessed these genes indicated the importance of these genes in bacterial pathogenesis. We suspected that there were other bacterial virulence factors or host factors that were involved in the pathogenesis of the fucose negative liver abscess KP strains. As fucose is part of the capsular components, disruption of fucose synthesis genes by mutation may contribute to the alteration of bacterial capsular glycotopes. Whether this mutation only will alter bacterial virulence remains to be investigated.

Possession of mucoid phenotype seemed not enough to confer virulence in mice. An *Escherichia coli* strain that was converted to mucoid phenotype by carrying a plasmid containing the KP genomic library genes did not show virulence in mice.[1] In addition, due to the heterogeneous content of the KP capsules, the exposed capsular epitope could be important in its association with host immune system. Subtle differences in bacterial capsular epitopes might confer different virulence in mice.

In conclusion, the virulent KpL1 contained capsule rich in fucose that was not seen in UTI-KP. The fucose synthesis genes were present in the virulent hepatic KpL1 and most of other hepatic-KP strains. This was different from the main terminal mannose found in KP causing UTI. The KpL1 did not interact with mouse peritoneal macrophage, while KpU1 was shown to interact with the macrophages. Intraperitoneal injection of KpL1 to nondiabetic mice displayed very high fatality, while KpU1 did not cause death in mice. The fucose in KpL1 could serve as immune evasion for bacteria to survive in host.

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