Infectivity of Hepatic Strain *Klebsiella pneumoniae* in Diabetic Mice

JUNE HSIEH WU[1] AND CHENG GIE TSAI

Department of Microbiology and Immunology, Graduate Institute of Basic Medical Science, School of Medicine, Chang Gung University, Taiwan

Besides urinary tract infection (UTI) and pneumonia, increased severe liver abscesses caused by *Klebsiella pneumoniae* (KP), especially in diabetic patients, have been observed in infections acquired in hospitals. This indicates that different KP strains with higher virulence have emerged in recent years. Our goal was to investigate the infectivity of KP isolates in mice from liver abscess or UTI patients. Mice were injected with streptozotocin to induce diabetes. Male ICR mice were infected with KpU1 (UTI strain CG3 for survival experiment only) and KpL1 (liver abscess strain CG5) by tail-vein injection of 5×10^4 colony-forming units (CFU) bacterial suspension. The mice survival rates, cytokine level by enzyme-linked immunosorbent assay (ELISA), and bacterial presence in liver tissue by Giemsa stain were examined. The survival rates for the KpL1-infected animals were 28% and 0% in normal and diabetic groups, respectively, whereas, for the KpU1-infected mice, the rates were 100% and 75% during a 30-day observation. Nonsurviving KpL1-infected mice showed $>10^5$ bacteria/ml blood and the bacteria appeared in the liver sinus area and inside liver cells. The KpL1-infected mice showed a tendency to increase the blood interleukin 1β (IL-1β) level in both nondiabetic and diabetic groups, whereas the tumor necrosis factor-α (TNF-α) level was significantly decreased in the KpL1-infected diabetic mice ($P = 0.002$). In conclusion, the KP strain from liver abscess showed a greater virulence in mice than the KP from UTI and was more virulent in diabetic than in nondiabetic mice. The infection with KP from liver abscess significantly decreased the blood TNF-α level in diabetes mellitus (DM) mice and the blood IL-1β level tended to increase in both infected nondiabetic and diabetic groups. High blood bacterial count and appearance of bacteria in liver sinus and cells usually contribute to death of the animals. Exp Biol Med 230:757–761, 2005

Key words: bacterial count; cytokines; diabetes mellitus; Giemsa stain; ICR mice; *Klebsiella pneumoniae*; liver abscess; survival rate

Introduction

Klebsiella pneumoniae (KP) is a gram-negative bacillus present in human intestinal tract as normal flora, but will cause disease in immunocompromised patients. Urinary tract infections (UTI) and pneumonia are most common clinical symptoms in KP infection. KP forms large mucoid colonies on culture plates and contains a thick capsule that is antiphagocytic. More than 80 capsular serotypes have been described. Capsular polysaccharide (CPS) of KP plays important role in gut colonization (1) and is an important virulence factor because KP with disrupted CPS production is avirulent to mice (2). Type I pili that are mannose-sensitive adhesion molecules can be expressed by KP (3). *Klebsiella pneumoniae* with defective CPS production expose pili and show greater adhesion to epithelial cells than wild-type KP (4). *Klebsiella pneumoniae* strains exposing mannose are recognized by macrophages and show low virulence; the bacteria are easily cleared from the blood of infected animals. *Klebsiella pneumoniae* with capsules, however, show low binding to macrophages and are highly virulent in mice (5).

Diabetes mellitus (DM) is a pathologic condition in that the metabolism of blood glucose is abnormal because of either insulin insufficiency or insulin receptor defect. Glucose level in blood is elevated with subsequent excretion in urine. High glucose level in blood can cause glycation of various cellular proteins. Formation of advanced glycation end products has been implicated in various diseases (6–9). Patients with DM have been reported to have an increased susceptibility to infections. Defect of IgG Fc fragment binding to protein A and complement has been observed in diabetes patients (10). Lower eradication rate was found for Helicobacter infection after antimicrobial therapy in diabetic patients than in nondiabetic controls (11), which may indicate an immune system defect associated with the diabetic state. Recent clinical observations of severe liver abscess in diabetic patients with KP infection could also be caused by emerging virulent bacterial strains. In this study, we examine the disease pattern caused by different KP strains, of UTI origin or liver abscess isolates, in diabetic and nondiabetic mice.

The work was supported by research grants CMRP1044 from Chang Gung University, Taiwan, and NSC 92-2320-B-182-052 and NSC 93-2320-B-182-024 from the National Science Council of Taiwan.

[1] To whom correspondence should be addressed at Department of Microbiology and Immunology, College of Medicine, Chang Gung University, 259 Wen Hua 1 Road, Kwei San, Tao Yuan 333 Taiwan. E-mail: jhwu@mail.cgu.edu.tw

Received April 26, 2005.
Accepted July 28, 2005.

1535-3702/05/23010-0757$15.00

Materials and Methods

Bacterial Strains and Animals. The KpL1 (CG5) strain was obtained through a clinical isolate from a diabetic patient with a liver abscess. The KpU1 (CG3) strain was obtained from a patient with a UTI. Both strains were tested for their serotype by *Klebsiella* antiserum kit (Denka Seiken, Tokyo, Japan). Neither KpL1 nor KpU1 were serotype K1. Only male ICR mice (*Mus musculus* CD-1) were used. 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. The male ICR mice were 6- to 8-weeks old and were purchased from Animal Center of National Taiwan University, Taipei, Taiwan. Mice were subsequently transferred and rehoused to the Animal Center, Chang Gung University. The mice were kept in a temperature of 21–23°C, a relative humidity of 50–70%, and a 12:12-hr light:dark cycle with normal chow feeding.

Induction of Diabetes in Mice. Mice were treated with intraperitoneal (ip) injection of 120 mg/kg of streptozotocin (STZ; Sigma, St. Louis, MO) dissolved in 0.05 *M* citrate buffer, pH 4.5. Control mice were treated with the same amount of citrate buffer without STZ. After 1 week, blood glucose level was measured, and, if the mice were not diabetic, a second dose of same amount STZ was again injected ip into the test group. In our experiment, the successful rate of diabetes induction after first STZ injection was approximately 50%. Blood glucose levels were measured 1 week after the second STZ injection. Blood glucose analysis used Glu-PIII (Fujifilm, Tokyo, Japan) measured on DRI-CHEM 3000 Colorimetric analyzer (Fujifilm). Mice with blood sugar levels >200 mg/dl were considered diabetic. All STZ-injected mice became diabetic in 3 weeks, in our observation. In the following experiments, the diabetic mice that were injected once or twice with STZ were evenly distributed in each group.

Infection of Mice With KP Bacteria for Survival Test. KP strains were cultured on blood agar plates overnight and scraped onto phosphate buffered saline (PBS; 0.8% NaCl, 0.02% KCl, 0.144% Na_2HPO_4, and 0.024% KH_2PO_4; pH 7.4) to make a bacterial suspension of 5×10^4 colony-forming units (CFU)/10 μl. Ten-microliter bacterial suspensions were injected into mice through the tail vein. Mice were divided into a Norm group ($n = 20$) for nondiabetic mice without KP infection; a DM group ($n = 21$) for diabetic mice without KP infection; a Norm-KpL1 group ($n = 18$) or a Norm-KpU1 group ($n = 10$) for nondiabetic mice infected with KpL1 or KpU1; and a DM-KpL1 group ($n = 27$) or a DM-KpU1 group ($n = 8$) for diabetic mice infected with KpL1 or KpU1. After infection, the mice were observed 30 days to determine survival rate.

Bacterial Count, Blood Cytokine Level, and Liver Tissue Histochemistry. A separate group of mice was used to obtain blood for cytokine analysis, bacterial count, and liver tissue sections. Only the liver abscess-KP (KpL1)–infected mice were studied for blood bacterial count, cytokine level, and bacterial presence in liver tissue sections. For blood cytokine levels, uninfected nondiabetic ($n = 7$), diabetic ($n = 8$) and KpL1-infected nondiabetic ($n = 25$) and diabetic ($n = 34$) mice were used. Mice were first anesthetized with ether before bleeding and surgery. Samples were obtained from both mice that survived and those that did not. Five microliters of blood was evenly spread on an Luria-Bertani agar (1% trypton, 0.5% yeast extract, 0.5% NaCl, and 1.5% agar) plate and incubated overnight before enumeration. Liver tissues were dissected, fixed in 10% formalin for 24 hrs, rinsed twice in PBS for 1 hr each, left in PBS for 24 hrs and were dehydrated in subsequent baths of 50%, 70%, 80%, 90%, 100%, 100%, and 100% ethanol for 40 min each, and finally immersed in xylene for 20 min. The tissue was embedded in 1:1 xylene/wax (Tyco Healthcare Group LP, Mansfield, MA) for 1 hr, in 1:2 xylene/wax for 24 hrs, and then in wax only to solidify into a tissue block. Sections from the tissue block were cut on a microtome (Micron International GmbH, Walldorf, Germany) to a thickness of 5 μm, and fixed on a glass slide.

Visualization of Bacteria by Giemsa Stain. For Giemsa staining, the sectioned tissue was rehydrated in subsequent immersions in xylene, xylene, then 95%, 75%, and 50% ethanol for 2 mins each, washed in distilled water, and stained in 1:20 diluted Giemsa stain (Sigma) solution for 30 mins and washed in distilled water to decolorize. The tissue was then sealed with mounting media (Merck KGaA, Darmstadt, Germany) and coverglass.

Detection of Cytokine Levels With Enzyme-Linked Immunosorbent Assay (ELISA). Cytokines TNF-α and IL-1β ELISA kits were purchased from Endogen (Pierce Biotechnology Inc. Rockford, IL). Experimental procedures followed the manufacturer's instructions. The cytokine level was determined from a standard curve performed at the same time with the test samples. Differences in levels were performed using Mann-Whitney Rank Sum test (12). A probability of less than 0.05 is considered statistically significant.

Results

The survival rate for the liver-KP–infected mice in the nondiabetes (Norm-KpL1) and diabetes (DM-KpL1) groups was 28% and 0%, respectively; whereas, for the UTI-KP strain infection, the survival rate was 100% and 75% for the nondiabetic mice (Norm-KpU1) and diabetic mice (DM-KpU1), respectively (Fig. 1). A trend of a higher blood glucose level that reduced the survival time of the liver-KP–infected mice was observed (Fig. 2). The blood bacteria count showed a biphasic curve, with a rise on 3rd and 5th day after infection, and a reduced count on the 4th day.

For the blood cytokine levels, there was a tendency for the infected group to show decreased TNF-β levels (Fig. 3A) and increased IL-1β levels (Fig. 3B). Except for the

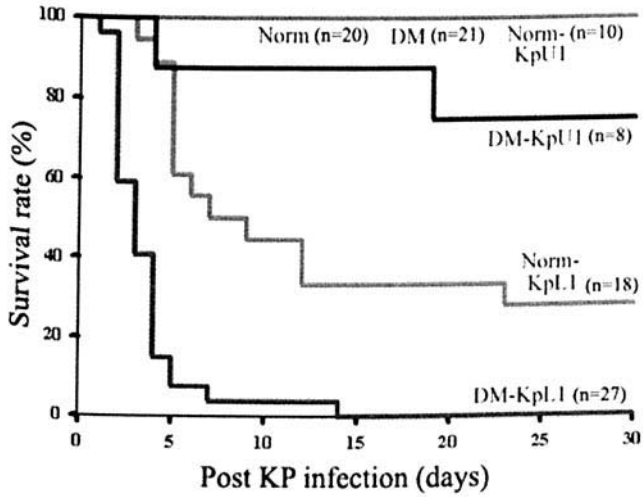

Figure 1. The survival curves of nondiabetic mice without KP infection (Norm), infected with KpL1 (Norm-KpL1), or KpU1 (Norm-KpU1); and diabetic mice without KP infection (DM), or infected with KpL1 (DM-KpL1), or KpU1 (DM-KpU1). The ICR mice were infected with 5×10^4 CFU KP through tail-vein injection, and observed for 30 days. Survival rates for Norm, DM, and Norm-KpU1 mice were 100%; for DM-KpU1 mice, 75%; for Norm-KpL1 mice, 28%; and for DM-KpL1 mice, 0%.

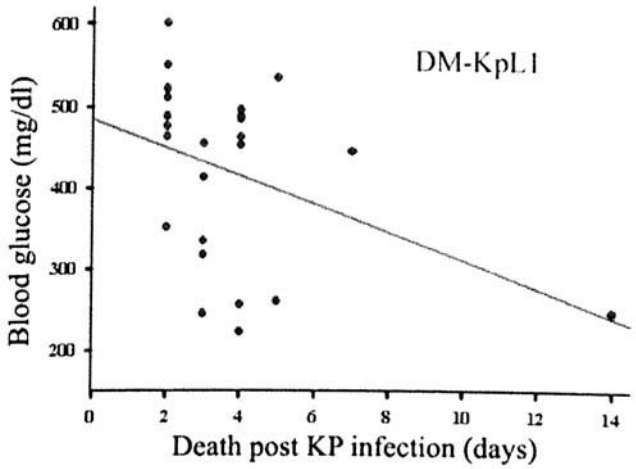

Figure 2. Relationship of blood-sugar levels and death after infection in diabetic mice infected with KpL1. Mice with high blood glucose levels tended to die earlier than mice with relatively lower blood glucose levels. Each dot represents one animal.

decrease in TNF-α level in the diabetic group, which reached statistical significance ($P = 0.002$) between the control and infected group, no statistically significant differences were observed for IL-1β level in Norm versus Norm-KpL1 ($P = 0.061$); or in DM versus DM-KpL1 ($P = 0.167$); or for TNF-α level in Norm versus Norm-KpL1 ($P = 0.207$).

Because liver section with hematoxylin and eosin stain was not able to show bacteria in the tissue, Giemsa stain was used for this purpose. Giemsa stain of the infected liver sections revealed many bacteria inside the capillary and sinus area (Fig. 4). Most bacteria could be seen clustered on the side of the capillary or sinus space. We also observed that mice with a blood bacteria count $>10^5$ CFU/ml did not survive 5 days after infection in the diabetic group, and many bacteria could be observed in the liver tissue and sinus area (Fig. 4A and B). Mice with a blood bacteria count $<10^4$ CFU/ml did not show any bacteria in the liver sinus area; however, in mice with a blood bacteria count between 10^4 and 10^5 CFU/ml, few bacteria could be observed in the large capillary area but not the sinus area (Fig. 4C and D).

Discussion

In the early 1970s, KP infection was not common. It usually involved patients' immunocompromised state and caused severe disease (13). Since 1970, multiple drug–resistant bacterial strains were found to be involved in hospital-acquired infections (14). In the past decade, KP has been found to cause bacteremia and severe liver abscess, although some KPs are still involved in UTI or pneumonia. Diabetes mellitus apparently exacerbates the disease condition. Our results in mice, showing the survival curve after

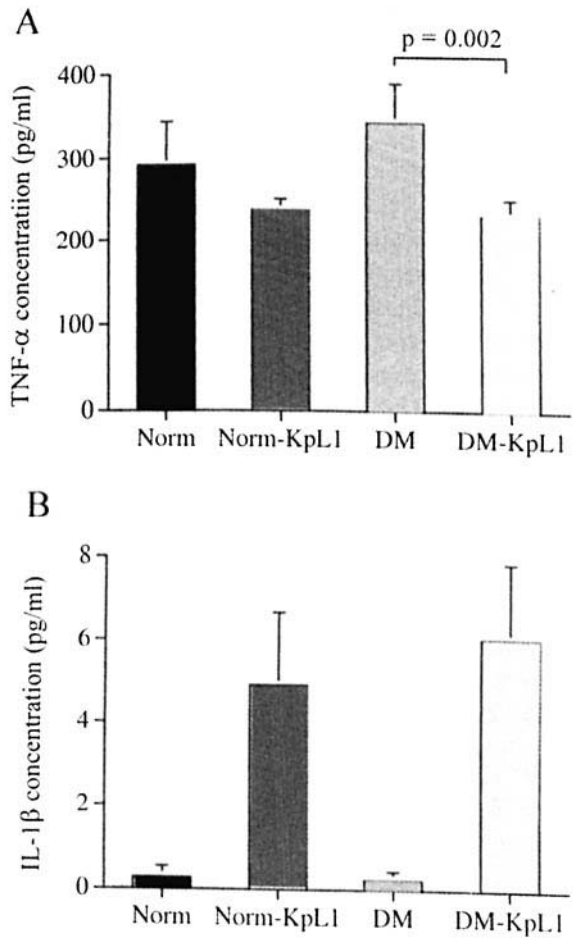

Figure 3. Blood levels of TNF-α (A) and IL-1β (B) in nondiabetic (Norm; $n = 7$) and diabetic (DM; $n = 8$) mice and infected nondiabetic (Norm-KpL1; $n = 25$) and diabetic (DM-KpL1; $n = 34$) mice. Vertical bars indicate standard error of the mean (SEM). (A) For TNF-α levels, the *P*-value between the Norm and Norm-KpL1 groups is 0.207; and between the DM and DM-KpL1 groups is 0.002. (B) For IL-1β levels, the *P*-value between the Norm and Norm-KpL1 groups is 0.061, and between the DM and DM-KpL1 groups it is 0.167.

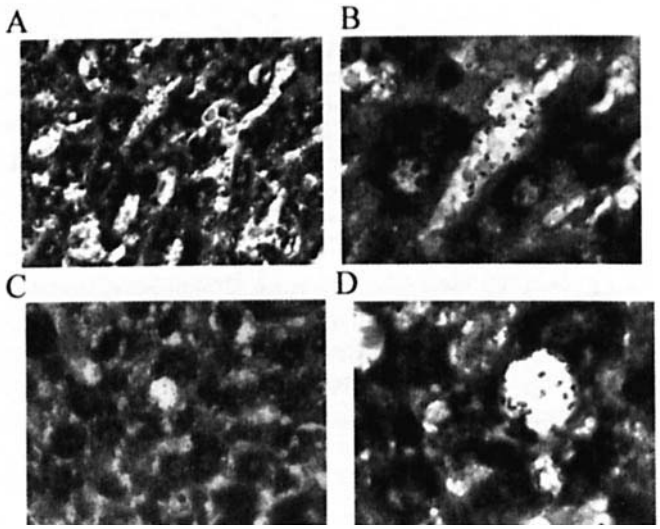

Figure 4. Giemsa stain of liver tissue section from diabetic mouse infected with KpL1. The mouse with blood bacteria count $>10^5$ CFU/ml showed many bacteria in liver tissue sinus area (A), higher magnification shown in (B). A mouse with a blood bacteria count between 10^4 and 10^5 CFU/ml showed bacteria in the capillary area, but rarely in the sinus area (C), higher magnification shown in (D).

infection with UTI-KP and liver abscess-KP strains illustrate that there is a difference in KP pathogenicity of different isolates. The liver abscess-KP strain not only causes rapid death in diabetic mice, it also causes a 72% death rate in nondiabetic mice. The UTI-KP strain causes only a 25% death rate in diabetic mice and no death in nondiabetic mice in a 30-day observation period.

Cortes *et al.* (2) infected ICR nondiabetic mice with 10^6 CFU KP by nose inoculation and found that the mice could survive at least 7 days. Their KP strain was isolated from pneumonia cases. Our initial pilot experiment, in which we used 10^6 CFU KP of liver abscess strain administered *via* tail vein injection, found that it was rapidly fatal in nondiabetic mice.

In human infections, KP first appears in blood, causing bacteremia before causing liver abscess; therefore, the infection route by tail vein was used in mice in our experiment to simulate human infection. In our experiment, infection with 10^2–10^4 CFU of KpL1 did not cause any symptoms in nondiabetic mice, and infection with 10^5 CFU induced rapid fatality in diabetic mice. The infectious dose of 5×10^4 CFU was therefore used in our experiment. Fang *et al.* (15) used infection doses of 10^2–10^5 CFU in BALB/c Byl mice through ip injection, and found that the infected nondiabetic mice died after 1 week, with liver abscess and meningitis. Our results differed from their results in the experiment involving nondiabetic mice, probably because of the different infection route and the different mouse strain.

For the cytokine levels, our data represented the mean of the cytokine concentrations 1–5 days after infection. No significant difference was observed in the mean value on a daily basis for each infected group. Yoshida *et al.* (16) used capsulated pneumonia-KP strain to infect nondiabetic male ICR mice by pulmonary inoculation, and found that the blood IL-1β and TNF-α concentrations were significantly increased 24 hrs after infection, and the rise in cytokine levels continued 48 hrs after infection. Our results on the infected diabetic and nondiabetic ICR mice, however, showed a different response on the TNF-α concentrations, although the IL-1β concentrations showed a similar trend. The difference may be caused by different KP strains and infection routes. The reason for the TNF-α downregulation, especially in the infected DM mice, is not known. Neutralization of TNF-α with anti–TNF-α antibody has been shown to severely impair the host defense against pathogens (17). Perhaps the liver abscess-KP downregulates the TNF-α concentration to suppress the immune response, because increased TNF-α can facilitate T-cell effector response and macrophage activation (18). The increase in blood IL-1β concentration may be involved in host defense mechanisms by activating kupffer cells and recruiting cellular components for defense. Interleukin-1β can also induce stress signals in liver parenchymal cells, leading to apoptosis or necrosis of the liver cells (19). The interplay of the cytokine regulation is complex, and bacteria may manipulate this regulation network to favor their survival in host tissues. Diabetes mellitus exacerbates the condition, because diabetics have been reported to have decreased IgG Fc function and complement fixation ability (10). Our mice survival test has demonstrated that the liver abscess-KP strain is particularly virulent. The virulence may reside on the surface capsule (2) or in some unknown pathogenicity genes in the liver abscess-KP strain. Our preliminary studies on the capsular surface of KpL1 and KpU1 revealed that KpL1 contains a capsule with surface antigens that are not found in the KpU1 strain (J.H. Wu, unpublished results). Perhaps similar to *Helicobacter pylori* (20), antigens on the bacterial surface are used by the microorganisms to evade host immune system. This may enhance the virulence of the organism.

In our observations, nonsurviving mice exhibited blood bacteria counts of $>10^5$, and many bacteria were observed in the liver sinus area near the sinusoidal walls. In these areas, gaps were present between endothelial cells (21), and direct contact of the blood with hepatocytes is a highly possible route for liver infection. We also observed KP inside the liver cells. However, apparent contact of the KP on the sinus wall with hepatocytes in the liver and the invasion of KP into hepatocytes in nonsurviving mice indicated that KP may initiate signaling that activates the genes, leading to liver abscess and lethality.

In conclusion, in ICR mice, KP of liver abscess isolate showed a higher virulence than the KP of UTI isolate; and a higher virulence in diabetic mice than in nondiabetic mice. The infection of the liver abscess-KP strain showed a tendency to increase blood IL-1β concentrations and the infection significantly decreased TNF-α levels in diabetic mice. Invasion of liver abscess KPs to the liver sinusoidal area indicates that the KP can multiply to high number in the

circulatory system without being contained by the host immune system.

1. Favre-Bonte S, Licht TR, Forestier C, Krogfelt KA. *Klebsiella pneumoniae* capsule expression is necessary for colonization of large intestines of streptomycin-treated mice. Infect Imm 67:6152–6156, 1999.
2. Cortes G, Borrell N, deAstorza B, Gomez C, Sauleda J, Alberti S. Molecular analysis of the contribution of the capsular polysaccharide and the lipopolysaccharide O side chain to the virulence of *Klebsiella pneumoniae* in a murine model of pneumonia. Infect Imm 70:2583–2590, 2002
3. Di Marino P, Bertin Y, Girardeau JP, Livrelli V, Joly B, Darfeuille-Michaud A. Molecular characterization and adhesive properties of CF29K, an adhesin of *Klebsiella pneumoniae* strains involved in nosocomial infections. Infect Imm 63:4336–4344, 1995.
4. Favre-Bonte S, Joly B, Forestier C. Consequences of reduction of *Klebsiella pneumoniae* capsule expression on interactions of this bacterium with epithelial cells. Infect Imm 67:554–561, 1999.
5. Kabha K, Nissimov L, Athamna A, Keisari Y, Parolis H, Parolis LAS, Grue RM, Schlepper-Schaefer J, Ezekowitz ARB, Ohman DE, Ofek I. Relationships among capsular structure, phagocytosis, and mouse virulence in *Klebsiella pneumoniae*. Infect Imm 63:847–852, 1995.
6. Basta G, Schmidt AM, De Caterina R. Advanced glycation end products and vascular inflammation: implications for accelerated altherosclerosis in diabetes. Cardiovasc Res 63: 582–592, 2004.
7. Mattson JS, Cerutis DR. Diabetes mellitus: a review of the literature and dental implications. Compendium of Continuing Education in Dentistry 22:757–760, 2001.
8. Schmidt AM, Stern DM. Receptor for age (RAGE) is a gene within the major histocompatibility class III region: implication for host response mechanisms in homeostasis and chronic disease. Frontiers in Biosci 6:D1151–1160, 2001.
9. Stitt AW. The role of advanced glycation in pathogenesis of diabetic retinopathy. Exp and Mol Pathol 75:95–108, 2003.
10. Dolhofer-Bliesener R, Lechner B, Gerbitz KD. Impaired immunoglobulin G Fc fragment function in diabetics is caused by a mechanism different from glycation. Eur J Clin Chem Clin Biochem 32:329–336, 1994.
11. Sargyn M, Uygur-Bayramicli O, Sargyn H, Orbay E, Yavuzer D, Yayla A. Type 2 diabetes mellitus affects eradication rate of *Helicobacter pylori*. World J Gastroenterol 95:1126–1128, 2003.
12. Kirkwood BR. Essentials of Medical Statistics. Oxford: Blackwell Scientific. pp150, 1988.
13. Fallon RJ. The relationship between the biotype of *Klebsiella* species and their pathogenicity. J Clin Pathol 26:523–528, 1973.
14. Cohen ML. Epidemiology of drug resistance: implications for a post-antimicrobial era. Science 257:1050–1055, 1992
15. Fang CT, Chuang YP, Chum CT, Chang SC, Wang JT. A novel virulence gene in *Klebsiella pneumoniae* strains causing primary liver abscess and septic metastatic complications. J Exp Med 199:697–705, 2004.
16. Yoshida K, Matsumoto T, Tateda K, Uchida K, Tsujimoto S, Yamaguchi K. Role of bacterial capsule in local and systemic inflammatory responses of mice during pulmonary infection with *Klebsiella pneumoniae*. J Med Microbiol 49:1003–1010, 2000.
17. Havell EA. Evidence that tumor necrosis factor has an important role in antibacterial resistance. J Immunol 143:2894–2899, 1989.
18. Dick AD, Forrester JV, Liversidge J, Cope AP. The role of tumour necrosis factor (TNF-alpha) in experimental autoimmune uveoretinitis (EAU). Prog Retinal Eye Res 23:617–637, 2004.
19. Hoek JB, Pastorino JG. Ethanol, oxidative stress, and cytokine-induced liver cell injury. Alcohol 27:63–68, 2002.
20. Wirth HP, Yang M, Karita M, Blaser MJ. Expression of the human cell surface glycoconjugates Lewis x and Lewis y by *Helicobacter pylori* isolates is related to cagA status. Infect Imm 64:4598–4605, 1996.
21. Weiss L. Histology Cell and Tissue Biology (5th ed.). New York: Elsevier Biomedical, pp724, 1977.