ORIGINAL ARTICLE

Impact of age on neutrophil phagocytic reaction with different capsular serotypes of *Klebsiella pneumoniae*

Chun-Hsiang Chiu [a], Kuo-Ming Yeh [b], Leung-Kei Siu [b], Chang-Phone Fung [c], Jung-Chung Lin [a,*], Feng-Yee Chang [a]

[a] Division of Infectious Diseases and Tropical Medicine, Department of Internal Medicine, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan
[b] Division of Clinical Research, National Health Research Institutes, National Yang-Ming University, Taipei, Taiwan
[c] Divison of Infectious Diseases, Taipei Veteran General Hospital, National Yang-Ming University, Taipei, Taiwan

Background: Although the prevalence of *K pneumoniae* liver abscess is higher in patients older than 55 years, the possible relationship of age with decreased phagocytic function of the patients with *Klebsiella pneumoniae* liver abscess has not been investigated. Our aim was to determine whether susceptibility to *K pneumoniae* infection depended on age-related impairment of phagocytic function.
Methods: The study enrolled 42 subjects in three age groups: younger than 40 years ($n = 10$), 40–65 years ($n = 12$), and older than 65 years ($n = 20$). Seventy-five strains of *K pneumoniae* were investigated, including liver abscess isolates ($n = 25$) and blood isolates from the patients without liver abscesses ($n = 50$). The rate of phagocytosis of K1/K2 ($n = 36$) and non-K1/K2 ($n = 39$) *K pneumoniae* by neutrophils was determined using flow cytometry and compared among the three age groups.
Results: The rate of phagocytosis of serotype K1/K2 isolates was significantly lower in the middle-aged group than that in the younger group ($p = 0.015$) and significantly lower in the older group than those in the middle-aged and younger groups ($p = 0.025$ and $p < 0.01$). In contrast, the rate of phagocytosis of non-K1/K2 isolates was similar in all three age groups at 60 minutes ($66.4 \pm 1.85\%$, $65.2 \pm 2.0\%$, and $62.3 \pm 1.81\%$; $p =$ not significant).

* Corresponding author. Department of Internal Medicine, Tri-Service General Hospital, 325, Section 2, Cheng-Kung Road, Neihu 114, Taipei, Taiwan.
  E-mail address: linjungchung1@yahoo.com.tw (J.-C. Lin).

Conclusions: Thus, as age increases, the ability of neutrophils to phagocytose virulent K1/K2 *K pneumoniae* decreases. This finding may account for the higher prevalence of *K pneumoniae* liver abscesses in older patients.

Introduction

Klebsiella pneumoniae causes suppurative infections, bacteremia, and a substantial percentage of nosocomial infections.[1,2] It is the most common cause of liver abscess in Taiwan, and the major predisposing factor is diabetes.[3,4] Although *K pneumoniae* liver abscess has been extensively studied, the pathogenesis is still not clear.

Diabetes depresses polymorphonuclear neutrophil functions, including adherence, chemotaxis, phagocytosis, and bactericidal activity and, thereby, increases the risk of vascular complications and infectious episodes.[5,6] The impairment of immunity is thought to be the cause of high prevalence of *K pneumoniae* liver abscess in patients with diabetes. To determine whether diabetes contributes to the development of *K pneumoniae* liver abscess, neutrophil phagocytic reaction with *K pneumoniae* in diabetic patients and normal healthy subjects were observed in our previous study.[7] It revealed that the rate of phagocytosis of K1/K2 *K pneumoniae* by neutrophils is significantly lower in patients with diabetes than in that in normal healthy subjects. No significant difference in the phagocytosis of non-K1/K2 *K pneumoniae* was observed among all subjects. Thus, serotype K1/K2 was considered to be an independent risk factor for the resistance of *K pneumoniae* to phagocytosis, and diabetic glycemic control was considered to have an additive effect.[7]

Otherwise, age is also considered an important factor of neutrophil phagocytic resistance against *K pneumoniae*. Two studies in Taiwan have shown that the median age of the patients with *K pneumoniae* liver abscesses was 61 years.[3,4] However, the impact of age on neutrophil phagocytic reaction with *K pneumoniae* has not been fully elucidated. To determine whether age impairs the phagocytic function of neutrophils, impeding the phagocytosis of K1/K2 strains of *K pneumoniae*, our present study compared the rate of neutrophil phagocytosis of K1/K2 and non-K1/K2 *K pneumoniae* strains by flow cytometry in three age groups of normal healthy subjects.

Methods

Subjects

Neutrophils were isolated from three age groups of subjects who were not immunocompromised and had no underlying diseases, such as malignancy, diabetes mellitus, and autoimmune diseases: a young group, that is, younger than 40 years ($n = 10$); a middle-aged group, that is, 40—65 years old ($n = 12$); and an old group, that is, older than 65 years ($n = 20$). The subjects (total $n = 42$) of the study were not infected within 4 weeks. Other 10 healthy volunteers were recruited for donating serum. The experimental procedures were reviewed and approved by the Ethics Committee of Tri-Service General Hospital, National Defense Medical Center, and informed consent was obtained from each participant.

Isolation of human neutrophils

Neutrophils were separated as follows. Heparinized blood (10—60 mL) was collected and mixed with an equal volume of dextran/saline solution. The mixture was allowed to sediment at room temperature for 40 minutes. The leukocyte-rich supernatant was layered over a density gradient of Ficoll-Paque (Pharmacia, Taipei, Taiwan). The samples were centrifuged at $400 \times g$ for 40 minutes at 20°C, the pellet was collected, erythrocytes were removed by hypotonic lysis, and isotonicity was restored using hypertonic saline. Each collected pellet was resuspended in ice-cold phosphate-buffered saline (PBS), and the cell concentration was adjusted to 1×10^7 cells/mL. Viability was greater than 95% by trypan blue exclusion.

Preparation of pooled serum

Pooled serum was prepared from 10 healthy volunteers after informed consent was obtained from each participant. Heparin-free blood drawn from the volunteers was clotted at room temperature and centrifuged ($1,000 \times g$ for 40 minutes at 20°C); the serum was removed, pooled, aliquoted, and stored at −70°C.

Collection of *Klebsiella pneumoniae* with different serotypes

Seventy-five *K pneumoniae* strains with different capsular serotypes were investigated, including liver abscess isolates ($n = 25$) and blood isolates from the patients without liver abscesses ($n = 50$) (Table 1). The serotypes were determined using the capsular swelling technique and countercurrent immunoelectrophoresis. *Klebsiella pneumoniae*

Table 1 Capsular serotype and source of *Klebsiella pneumoniae* that were selected for phagocytosis assay

Serotypes	Source of isolate	
	Liver abscess	blood
K1	16	9
K2	5	6
Non-K1/K2[a]	4	35
Total	25	50

[a] Serotypes included K3, K5, K6, K7, K8, K9, K15, K16, K17, K20, K21, K28, K29, K31, K32, K38, K54, K55, and K57.

strain DT-S (biotype *edwardsii*, capsular serotype K1) and its acapsular mutant of DT-X were kindly provided by Takeda Pharmaceuticals, Osaka, Japan. DT-S was derived from *K pneumoniae* DT isolated from the sputum of a patient with pneumonia. The lack of a capsule by DT-X was confirmed using India ink staining. Both DT-S and DT-X were stored at −80°C.

Fluorescence labeling of bacteria

Klebsiella pneumoniae isolates were incubated overnight at 37°C. The cell concentration was adjusted spectrophotometrically (Olympus, New York, USA) and confirmed by quantitative colony counts. Bacteria were killed by heating for 60 minutes in a 70°C water bath. The bacteria were washed with PBS and labeled with fluorescein isothiocyanate [FITC (0.1 mg/mL); Sigma Chemical Co., St. Louis, MO, USA] in 0.10M NaHCO$_3$, pH 9.0, for 60 minutes at 25°C. FITC-labeled bacteria were resuspended to 2×10^8 cells/mL of PBS, aliquoted, and stored at −70°C. The aliquots were thawed before use.

Phagocytosis reaction

Phagocytosis was measured by using a standard assay.[8] FITC-labeled bacteria [200 μL; 4×10^7 colony-forming units (CFU)/mL] were added to a prewarmed mixture (shaken for 5 minutes at 37°C) of 100 μL of neutrophil suspension (i.e. 1×10^6 cells); 100 μL of freshly thawed pooled normal human serum (10% vol/vol; used for opsonization); and 600 μL of PBS in 10 × 75 mm polypropylene tubes (BD, Franklin Lakes, NJ, USA). The tubes were agitated continuously for 2 minutes, 5 minutes, 10 minutes, 30 minutes, and 60 minutes. Each strain was reactivated with neutrophils donated from three different normal healthy subjects.

Phagocytosis assay using flow cytometry

FITC fluorescent neutrophils were analyzed using a FACScan with an argon ion laser (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) as previously described.[8] A total of 10,000 neutrophils were processed using the Cellquest version 1.0 software (Becton Dickinson Immunocytometry Systems). By analyzing the mixtures of labeled and unlabeled bacteria, the boundary between positive and negative fluorescence was determined. The percentage of ingested bacteria was assessed after the addition of ethidium bromide.

Statistical analysis

Between-group differences in the rate of neutrophil phagocytosis over time were examined by means of one-way analysis of variance with repeated measures. Between-group differences in the rate of neutrophil phagocytosis at 60 minutes were assessed by the Student's *t* test. Pearson's correlation was used to evaluate the relationship between age and rate of phagocytic uptake. Differences were considered to be significant at *p* values less than 0.05; all statistical tests were two sided. Data were presented as mean ± standard error of the mean.

Results

To differentiate between age and capsular effects, *K pneumoniae* were grouped into serotypes K1/K2 and non-K1/K2. Figures 1 and 2 compared the rate of neutrophil phagocytosis in three age groups. The rate of K1/K2 phagocytosis was significantly lower in the middle-aged group than the young group ($p = 0.015$) and significantly lower in the old group than the middle-aged and young groups ($p = 0.025$ and $p < 0.01$) (Fig. 1). Overall, the trend toward decreased rate of phagocytosis with age appeared to be much more significant for K1/K2 *K pneumoniae* isolates than for non-K1/K2 isolates. No significant difference in the rates of phagocytosis of non-K1/K2 isolates was observed between the young and old groups at 60 minutes ($66.4 \pm 1.85\%$ and $65.2 \pm 2.0\%$; $p = 0.69$); the middle-aged and old groups ($65.2 \pm 2.0\%$ and $62.3 \pm 1.81\%$; $p = 0.49$); and old and young groups ($62.3 \pm 1.81\%$ and $66.4 \pm 1.85\%$; $p = 0.13$) (Fig. 2).

Phagocytosis of serotype K1/K2 isolates declined with age and was significantly impaired in neutrophils from subjects older than 65 years. The impairment was already obvious at 10 minutes and persisted upto 60 minutes. Pearson's correlation analysis of the relationship of phagocytosis with age found that for K1/K2 strains, the rate of phagocytic uptake decreased significantly with increasing age ($r = -0.452$, $p < 0.01$) (Fig. 3), whereas no such relationship was found for non-K1/K2 strains (Fig. 4).

The rate of phagocytosis of the encapsulated strain of *K pneumoniae* DT-S and its nonencapsulated counterpart DT-X were compared in the three age groups. The percentage of strain DT-S ingested by neutrophils decreased with age from $44.0 \pm 2.7\%$ (in the young group) to $37.0 \pm 4.5\%$ and $34.0 \pm 1.3\%$ (in the middle-aged and old groups, respectively) and was comparable to decreases caused using multiple strains of K1/K2 *K pneumoniae*. In contrast, the ingested percentage of DT-X strain was similar in all three groups ($80.0 \pm 3.0\%$, $82.2 \pm 3.7\%$, and $82.3 \pm 2.4\%$, respectively; Fig. 5). Thus, the capsule of *K pneumoniae* may play an important role in the age-related decrease in phagocytic function.
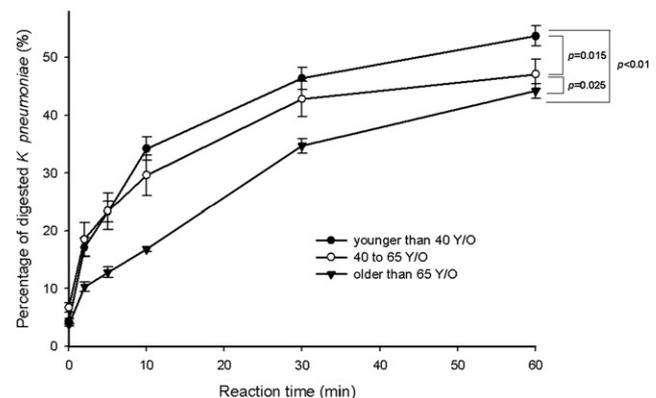
Figure 1. Comparisons of neutrophil phagocytosis of serotype K1/K2 isolates among different age groups of younger than 40 years, 40—65 years, and older than 65 years. Y/O = years old.
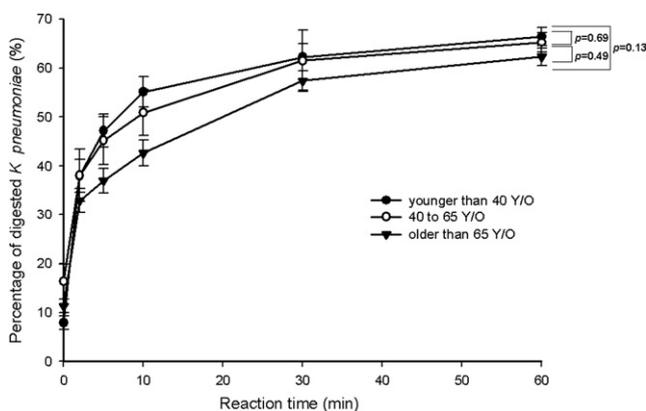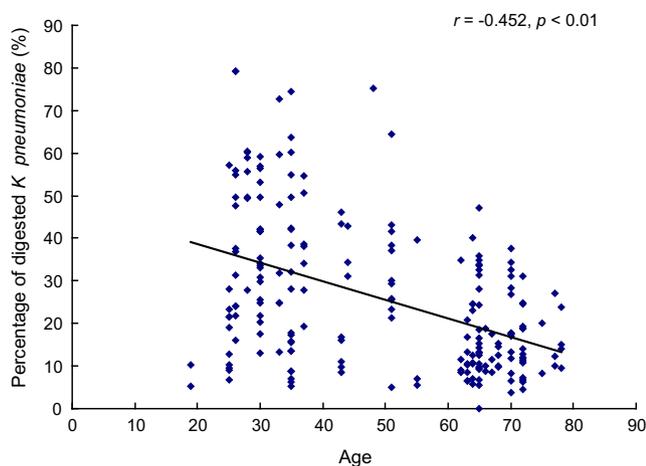
Figure 2. Comparisons of neutrophil phagocytosis of non-K1/K2 isolates among different age groups of younger than 40 years, 40–65 years, and older than 65 years. Y/O = years old.
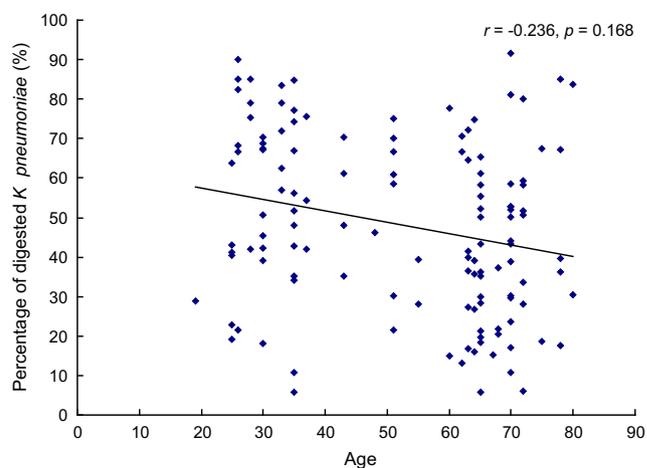
Discussion

Our study demonstrated that advancing age is accompanied by a decreased ability of neutrophils to phagocytose *K pneumoniae*. The age-related impaired phagocytosis is statistically significant for K1/K2 *K pneumoniae* but not for non-K1/K2 strains. Similarly, an age-related decline in the rate of phagocytosis of DT-S but not of its isogenic mutant strain (DT-X) was also demonstrated.

The immune system has innate and adaptive components. Age-related changes in the immune system (also known as immunosenescence) may increase the susceptibility of the elderly to infectious diseases and possibly to autoimmune diseases and cancer.[9] Previous studies have demonstrated that both innate and adaptive immunity will be dysregulated as age increases. However, it appears that adaptive immunity is more susceptible than innate immunity to these age-dependent changes.[10] The adaptive immune system of T and B cells can provide memory of

Figure 4. Comparison of neutrophil phagocytosis of non-K1/K2 *Klebsiella pneumoniae* at 10 minutes among patients of different age groups.

a previous infection that enables a more rapid response to subsequent encounters with the same pathogen. Because of the age-related involution of the thymus, the number of T cells exiting the thymus decreases significantly with age.[11] The functions of the T cells and B cells also decline dramatically with age.[12–15]

Epithelial barriers (mucosa, neutrophils, macrophages, natural killer cells, dendritic cells, cytokines, and chemokines) play an important role in innate immunity, which is the first line of host defense. With age, the function of the respiratory and gastrointestinal tract mucosal barriers decreases, enabling pathogens to invade the mucosa.[16]
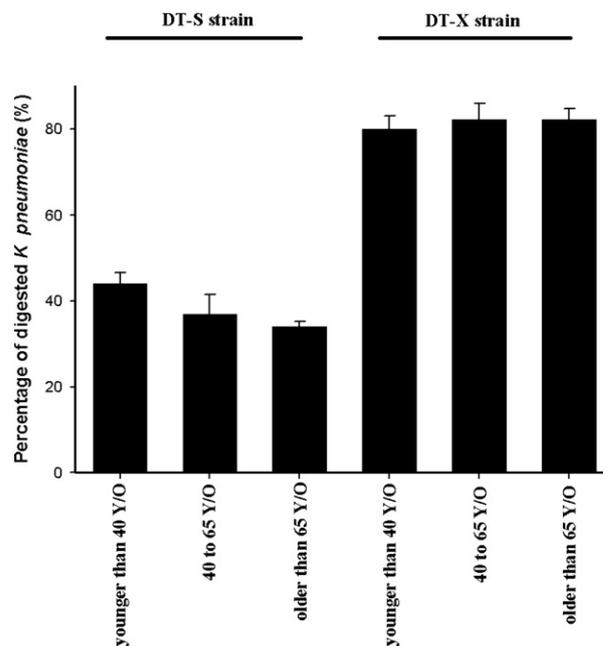

Figure 3. Comparison of neutrophil phagocytosis of K1/K2 *Klebsiella pneumoniae* at 10 minutes among patients of different age groups. Pearson correlation test was used to evaluate the correlation between age and phagocytic uptake rate.

Figure 5. Comparisons of neutrophil phagocytosis of serotypes K1 (DT-S) and isogenic mutant with acapsular serotype K1 isolates (DT-X) among different age groups of younger than 40 years, 40–65 years, and older than 65 years. Y/O = years old.

Neutrophils are the first inflammatory cells recruited in response to inflammation or tissue infection. Previous studies have shown no difference among age groups in endothelial adherence, migration, and secretory granule activity, although aging reduced the delivery of neutrophils *in vivo* into skin abrasions[17] and reduced the function of neutrophils in patients with chronic bronchitis and poorly controlled diabetes.[18,19] In patients with diabetes, aging decreased the phagocytosis of K1/K2 *K pneumoniae* but had no effect on the phagocytosis of non-K1/K2 *K pneumoniae*. Our study found that not only host factors but also pathogenicity of the bacterial species influence age-related change in the rate of phagocytosis. Reduced innate immunity may explain the increased susceptibility of the elderly to infection, as exemplified by increased incidence of *K pneumoniae* liver abscess in elderly patients with median age of 56.5–69 years.[20,21]

Our previous study demonstrated that K1/K2 strains are more resistant than non-K1/K2 strains to phagocytosis in both normal healthy subjects and patients with Type 2 diabetes.[7] It had been reported that capsular polysaccharide protects *K pneumoniae* from the bactericidal action of serum and from the phagocytosis and killing of neutrophils.[22–25] Thus, we supposed that capsular polysaccharide also plays an important role in the different results of the impact of age on neutrophil phagocytic reaction with different capsular serotypes of *K pneumoniae*. On comparing the neutrophil phagocytosis of different strains—encapsulated K1 *K pneumoniae* (DT-S) and nonencapsulated K1 *K pneumoniae* (DT-X)—we found an age-related decline in the rate of phagocytosis of DT-S but not of its isogenic mutant strain DT-X. Thus, capsular polysaccharides protect K1/K2 *K pneumoniae* from neutrophil phagocytic reaction and lead to greater phagocytic resistance in aged people.

In conclusion, our study demonstrated that (1) the ability of neutrophils to phagocytose K1/K2 *K pneumoniae* is decreased (but the ability to phagocytose non-K1/K2 is not) and (2) the rate of phagocytosis of K1/K2 (but not non-K1/K2) *K pneumoniae* declines with age.

Acknowledgments

References

1. Yinnon AM, Butnaru A, Raveh D, Jerassy Z, Rudensky B. *Klebsiella* bacteraemia: community versus nosocomial infection. *QJM* 1996;**89**(12):933–41.
2. Montgomerie JZ. Epidemiology of *Klebsiella* and hospital-associated infections. *Rev Infect Dis* 1979;**1**(5):736–53.
3. Chang FY, Chou MY. Comparison of pyogenic liver abscesses caused by *Klebsiella pneumoniae* and non- *K. pneumoniae* pathogens. *J Formos Med Assoc* 1995;**94**:232–7.
4. Tsai FC, Huang YT, Chang LY, Wang JT. Pyogenic liver abscess as endemic disease, Taiwan. *Emerg Infect Dis* 2008;**14**(10):1592–600.
5. Tsay RW, Siu LK, Fung CP, Chang FY. Characteristics of bacteremia between community-acquired and nosocomial *Klebsiella pneumoniae* infection: risk factor for mortality and the impact of capsular serotypes as a herald for community-acquired infection. *Arch Intern Med* 2002;**162**:1021–7.
6. Delamaire M, Maugendre D, Moreno M, Le Goff MC, Allannic H, Genetet B. Impaired leucocyte functions in diabetic patients. *Diabet Med* 1997;**14**:29–34.
7. Lin JC, Siu LK, Fung CP, Tsou HH, Wang JJ, Chen CT, et al. Impaired phagocytosis of capsular serotypes K1 or K2 *Klebsiella pneumoniae* in type 2 diabetes mellitus patients with poor glycemic control. *J Clin Endocrinol Metab* 2006;**91**(8):3084–7.
8. Heinzelmann M, Gardner SA, Mercer-Jones M, Roll AJ, Polk Jr HC. Quantification of phagocytosis in human neutrophils by flow cytometry. *Microbiol Immunol* 1999;**43**(6):505–12.
9. Pawelec G. Immunosenescence: impact in the young as well as the old? *Mech Ageing Dev* 1999;**108**(1):1–7.
10. Franceschi C, Bonafe M, Valensin S. Human immunosenescence: the prevailing of innate immunity, the failing of clonotypic immunity, and the filling of immunological space. *Vaccine* 2000;**18**(16):1717–20.
11. Fagnoni FF, Vescovini R, Passeri G, Bologna G, Pedrazzoni M, Lavagetto G, et al. Shortage of circulating naïve CD8(+) T cells provides new insights on immunodeficiency in aging. *Blood* 2000;**95**:2860.
12. Chakravarti B, Abraham GN. Aging and T-cell-mediated immunity. *Mech Ageing Dev* 1999;**108**:183–206.
13. Hodes RJ, Fauci AS, editors. *Report of task force on immunology and aging*. Bethesda, MD: National Institutes of Aging and Allergy and Infectious Diseases, US Department of Health and Human Services; 1996.
14. Cossarizza A, Ortolani C, Monti D, Franceschi C. Cytometric analysis of immunosenescence. *Cytometry* 1997;**27**:297–313.
15. Burns EA, Goodwin JS. Immunodeficiency of aging. *Drugs Aging* 1997;**11**:374–97.
16. Gomez CR, Boehmer ED, Kovacs EJ. The aging innate immune system. *Curr Opin Immunol* 2005;**17**(5):457–62.
17. MacGregor RR, Shalit M. Neutrophil function in healthy elderly subjects. *J Gerontol* 1990;**45**(2):M55–60.
18. Hirokawa K. Immunity and aging. In: Pathy MSJ, editor. *Principles and practice of geriatric medicine*. 3rd ed. New York: John Wiley and Sons; 1998. p. 35–47.
19. Rassias AJ, Marrin CA, Arruda J, Whalen PK, Beach M, Yeager MP. Insulin infusion improves neutrophil function in diabetic cardiac surgery patients. *Anesth Analg* 1999;**88**(5):1011–6.
20. Pastagia M, Arumugam V. *Klebsiella pneumoniae* liver abscesses in a public hospital in Queens, New York. *Travel Med Infect Dis* 2008;**6**(4):228–33.
21. Hui JY, Yang MK, Cho DH, Li A, Loke TK, Chan JC, et al. Pyogenic liver abscesses caused by *Klebsiella pneumoniae*: US appearance and aspiration findings. *Radiology* 2007;**242**(3):769–76.
22. Hall HE, Humphries JC. The relationship between insusceptibility to phagocytosis and virulence of certain *Klebsiella pneumoniae* strains. *J Infect Dis* 1958;**103**:157–62.
23. Pruzzo C, Guzman CA. Efficient phagocytosis of *Klebsiella pneumoniae* strains that poorly bind to human polymorphonuclear leukocytes. *Int Microbiol* 1998;**1**:53–7.
24. Podschun R, Teske E, Ullmann U. Serum resistance properties of *Klebsiella pneumoniae* and *K. oxytoca* isolated from different sources. *Zentralbl Hyg Umweltmed* 1991;**192**:279–85.
25. Kabha K, Nissimov L, Athamna A, Keisari Y, Parolis H, Parolis LA, et al. Relationships among capsular structure, phagocytosis, and mouse virulence in *Klebsiella pneumoniae*. *Infect Immun* 1995;**63**:847–52.