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A SURVEY OF *LEGIONELLA PNEUMOPHILA* AMONG PNEUMONIA PATIENTS AT KENYATTA NATIONAL HOSPITAL
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ABSTRACT

Objective: **To determine the occurrence of *L. pneumophila* among pneumonia patients at Kenyatta National Hospital and any association with possible risk factors.**
Design: **A cross- sectional descriptive study.**
Setting: **The study was conducted from March to June 2007, at the medical ward of Kenyatta National Hospital. Analysis of samples was done at the University of Nairobi Institute of Tropical and Infectious Diseases (UNITID) serology laboratories.**
Subjects: **All adult patients who were admitted to the medical ward of the hospital with a provisional diagnosis of pneumonia.**
Results: **The study indicated that up to 9.2% (11 out of 120) of the pneumonia patients admitted at the hospital were infected with *L.pneumophila*. At a confidence limit of 0.05, there was statistical significance in the number of pneumonia patients infected with *L. pneumophila* and exposure to air conditioners (p= 0.003). Twenty two point five eight per cent of patients who were exposed to air conditioners were positive for *L. pneumophila* urinary antigen. There was a statistical significance between exposure to air conditioners and location of work area (p= 0.001)). Thirty eight point four six per cent of those who worked indoors were exposed to air conditioners at their places of work. There was also statistical significance in the number of pneumonia patients infected with *L. pneumophila* and a history of a past or concurrent respiratory illness (p= 0.021).**
Conclusion: **Exposure to air conditioners and a history of past or concurrent respiratory illness were found to predispose one to infection. This should raise the index of suspicion among clinicians as they obtain a patient's medical history. Most of those exposed to air conditioners are exposed at their places of work in urban centres, hence the need for health education and public awareness on routine inspection and maintenance of such facilities. There is need for a larger multi-centre study on the prevalence of infection by *L. pneumophila* in pneumonia patients (both community acquired and nosocomial), existence of co- infection and the antibiotic susceptibility of isolated organisms.**

INTRODUCTION

Since the epidemic of legionnaires disease in Philadelphia in 1976 and the description of the causative agent *Legionella pneumophila*, there have been several reports of outbreaks and single cases throughout the world (1).

Legionella pneumophila is recognised as one of the causes of atypical pneumonia, both community acquired and nosocomial (2). The incidence of community acquired legionnaires disease varies widely according to the setting investigated and the diagnostic methodology applied. Since many countries lack appropriate methods of diagnosing the infection or surveillance systems capable of monitoring the situation, the real magnitude of the problem is unknown and it may be responsible for more of the pneumonia occurring in the tropics than is generally recognised. Infections of any kind can be recognised more accurately with increased physician awareness and availability of diagnostic tools. Serological tests are mainly applied as epidemiological tools and can only be useful in diagnosis of disease when the background prevalence of antibody to *L. pneumophila* within the local community is established thereby providing a correct guide for interpretation of serological tests. However reliability of serological testing is hampered

by several limitations including cross reactions due to antibodies to *Pseudomonas aeruginosa* and *Campylobacter spp* (3,4). The need for testing of paired serum samples collected three to six weeks apart also diminishes the use of antibody testing in serology tests. Urine antigen is now the most frequently used diagnostic test permitting early diagnosis, initiation of appropriate therapy and a rapid public health response (5).

Prevalence surveys have been conducted in several countries all over the world. In the developed countries concrete systems of surveillance of *legionella* in the environment and monitoring of reports of *legionellosis* outbreaks have been established through the Centres for Disease Control and Prevention (in USA) and the European Working Group for *Legionella* Infections (EWGLI) (6). The disease is a major concern of public health professionals and individuals involved in maintaining building water systems. Studies have estimated that between 8000 and 18000 persons are hospitalised with *legionellosis* annually in the United States of America (2). In 2003, 34 countries (population: 467.76 million) out of the 36 in the EWGLI reported a total of 4578 cases, meaning an average rate across Europe of 9.8 per million population. Reports of legionnaire's disease in developing countries especially from Africa have been sparse. Studies of antibody prevalence across the continent are few and *legionellosis* is hardly considered during differential diagnosis of respiratory infections. Failure to diagnose *legionellosis* is largely due to lack of clinical awareness.

The factors that lead to outbreaks or cases of legionnaires' disease are not completely understood, but certain events are considered prerequisites for infection. These include the presence of the bacterium in an aquatic environment, amplification of the bacterium to an unknown infectious dose, and transmission of the bacteria to a human host that is susceptible to infection (1).

Kenya has witnessed an expansion of its urban centres in the last decade, with increased usage of facilities such as air conditioners and hot water systems both of which are the main man-made habitats of *legionella*. Factors that have been observed in other places to predispose one to infection with *legionella* such as cigarette smoking are also present in our setting making it possible that legionellosis could be an under-reported and under-diagnosed disease. The aim of this study was to investigate what proportion of pneumonia patients at the medical ward are excreting *L. pneumophila* antigen and evaluating for possible risk factors in the positive group using demographic and clinical data. The study also generated useful data on socio-demographic factors of pneumonia patients in general.

Ethical issues: Informed consent was obtained from the participants. Approval to carry out the study was obtained from the Kenyatta National Hospital Research and Ethics Committee, and the Chairman of the Department of Internal Medicine, Kenyatta National Hospital. The approval was on the agreement that participants anonymity must be maintained, good laboratory practice/quality control ensured, and that every finding would be treated with utmost confidentiality and for the purpose of this research only. The clinicians were notified of positive findings for consideration during treatment.

MATERIALS AND METHODS

Study design: This was a cross-sectional descriptive study which entailed consecutive sampling of patients admitted with a provisional diagnosis for pneumonia and investigating for the presence or absence of infection with *legionella*. Their demographic and clinical data were also evaluated.

Study site: The study was conducted at the medical wards of Kenyatta National Hospital, a university affiliated hospital located in Nairobi, Kenya. It is a primary and secondary health care facility. Analysis of samples was done at the University of Nairobi Institute of Tropical and Infectious Diseases (UNITID) serology laboratories.

Study population: The study population comprised of adult (above 18 years) patients admitted with pneumonia at the medical wards of the hospital. Urine samples were obtained from patients who consented to the study. Demographic data and other vital patient details were obtained using questionnaires and patient files.

Sample size: A total of 120 patients were selected for the study as estimated using the formular by Fisher *et al* (7). Based on a survey done in South Africa (8), a prevalence of 8.7% was used in calculation of the sample size.

Sampling: All patients who were admitted at the medical wards with a provisional diagnosis for pneumonia and satisfied the inclusion criteria were recruited into the study by consecutive sampling. This entailed sampling every patient who met the defined eligibility criteria until the predetermined sample size was achieved.

Inclusion criteria: All adult patients (above 18 years), hospitalised due to initial presumptive diagnosis of pneumonia were eligible for the study. The patients who consented to the study were recruited and urine samples obtained within three days of admission.

Study limitations: Some of the patients had difficulties in recalling or expressing information. The researcher

would have liked to expand the study area to include patients from other hospitals but due to limited time and resources, this was not possible.

Data collection: A structured questionnaire written in English was administered to collect demographic and qualitative data from study subjects. Patients who did not understand English were questioned in Kiswahili. One research assistant assisted in administering the questionnaires. Information obtained from the medical records was filled out on the clinical data forms. The laboratory results for each patient were recorded on the questionnaire.

Specimen collection: Urine samples were collected in standard sterile containers (universal bottles) and transported to the laboratory within one hour. The urine was then aliquoted into smaller sterile containers, coded and frozen at -20°C.

Laboratory methods: Before processing the urine was thawed and warmed to 37°C using a water bath. The urine was then analysed for the presence of *L. pneumophila* urinary antigen using an enzyme linked immunosorbent assay (ELISA) procedure. The kit that was used was the Binax Legionella urinary antigen enzyme immunoassay (9). It is a test system intended for *in-vitro* diagnostic use to qualitatively detect the presence of *legionella pneumophila* serogroup 1 antigen. It uses the microtiter ELISA methodology for the detection of soluble antigen in urine from patients with *Legionella pneumophila* serogroup 1 infections.

Principle of the test: The strips of a microplate are coated with polyclonal rabbit antibody which reacts with *L. pneumophila* antigen. Patient urine was added to the wells of the microplate and any *legionella* antigen present bound to the specific antibody at the solid phase. Following the first incubation, the wells were washed and a peroxidase-labelled antibody, which reacts with *L. pneumophila* antigen, was added and it binds to free binding sites on the antigen during a second incubation. After a further washing stage, the presence of bound peroxidase was demonstrated in a colour reaction with a substrate. The reaction was stopped by adding sulphuric acid and the optical density measured with a spectrophotometer at 450nm. The performance characteristics of this test have been evaluated by the manufacturer (9). It has a sensitivity of 97.7% and a specificity of 100%.

Calculation and interpretation of results: The absorbance of the substrate blank was subtracted from the absorbance of each well, to obtain a corrected absorbance for use in the calculation of patient results. Positive and negative controls were run in duplicate therefore a mean absorbance was used in calculation of the ratios. The ratio was determined by dividing the absorbance of the patient's urine with the mean absorbance of the negative control. Urine samples that had a ratio value greater than or equal to three were considered positive for the presence of *L. pneumophila* serogroup 1 antigen. Ratio values of less than three were considered negative.

Quality control:

(i) All samples were obtained within three days of admission.
(ii) Laboratory data were generated using the proper protocol and standards to ensure effectiveness.
(iii) Positive and negative urine controls supplied in the kit were run in duplicate in every batch of tests.
(iv) The average absorbance of all negative controls fit the required criteria of an absorbance of less than or equal to 0.100.
(v) The average absorbance of all positive controls also fit the required criteria of greater than or equal to three times that of the negative controls.
(vi) Urine samples from 17 patients admitted in the medical wards for conditions other than pneumonia were also included as controls. All of them tested negative.
(vii) Coding of data were done accurately

Data management: A computer based file was developed using SPSS. The results were then presented in descriptive statistics using frequency tables, cross tabulation and pie charts. Frequencies of various parameters were obtained. Chi-square and Fishers' Exact tests for significance were used to analyse the risk factors. The level of significance for this study had been set at 0.05.

RESULTS

Out of a total of 120 respondents 11 tested positive for *L. pneumophila* serogroup 1 antigen in their urine, revealing that 9.2% of pneumonia patients admitted at the medical wards of Kenyatta National Hospital between March and June 2007 were infected with *L. pneumophila* serogroup 1. Positive cases were identified by using the optical density readings to calculate ratio values (Figures 1 and 2). Samples 16, 59, 70, 71, 77, 87, 89, 92, 106, 109, 111 that were processed in microtitre plates 1 and 3 showed a ratio value of more than three.

Figure 1
Binax legionella urinary antigen microplate 1: Calculated ratio values

	1	2	3	4	5	6	7	8	9	10	11	12
A		2.69	2.11	1.57								
B	1.0	1.45	1.99	1.77								
C	1.0	2.02	2.21	2.25								
D		1.45	2.06	1.09								
E		2.24										
F	1.49	2.83	1.87									
G	2.33	1.71	1.23									
H	1.47	2.60	1.29									

Key
Positive for *L. pneumophila antigen*
Negative for *L. pneumophila antigen*
Empty wells
Ratio values = Mean positive control OR patient sample absorbance
Mean negative control absorbance
Interpretation: >3 presumptive positive for the presence of *L. pneumophila* serogroup 1 antigen in urine, suggesting current or past infection.
<3 presumptive negative for *L. pneumophila* serogroup 1 antigen in urine, suggesting no recent or current infection.
Positive samples: E3- 1 sample- Sample 16
D and E = Positive control
B and C = Negative control

Figure 2
Binax legionella urinary antigen microplate 3: Calculated ratio values

	1	2	3	4	5	6	7	8	9	10	11	12
A		0.8	0.91	0.87	1.59	1.76	1.07	1.3	1.15	2.6		0.52
B	1.0	1.79	1.66	0.98	2.0	2.24		1.64	1.48	2.64	1.98	0.78
C	1.0	1.21	1.21	0.77	1.49		2.98	1.67		2.67	2.5	2.62
D		1.71	0.87	1.45	1.45		1.21		2.5	1.89		0.9
E		2.24	0.98	1.66	1.66	2.1	1.2	1.84	1.15	1.55	2.48	1.67
F	0.8	0.98	1.39	1.63	1.51	1.56	1.77	2.46	2.10	1.69	4.9	0.86
G	1.1	1.23	1.69		2.14	1.79	1.67	6.91	2.14	1.15	1.23	0.98
H	2.02	1.69	1.07	1.29	1.21	1.63	1.45	1.35	1.24	2.7	2.42	1.05

Key
Positive for *L. pneumiphile antigen*
Negative for *L. pneumophila antigen*
Empty wells
Ratio values = Mean positive control OR patient sample absorbance
Mean negative control absorbance
Interpretation: >3 presumptive positive for the presence of *L. pneumophila* serogroup 1 antigen in urine, suggesting current or past infection.
<3 presumptive negative for *L. pneumophila* serogroup 1 antigen in urine, suggesting no recent or current infection.

All non- pneumonia patients admitted to the medical ward but included in the study as controls tested negative for the antigen. All positive and negative kit controls fitted the required criteria for quality control.

A summary of demographic, socio-economic and clinical characteristics of the respondents have been shown in Table 1. The frequencies of socio demographic factors obtained were cross-tabulated with the occurrence of *L. pneumophila* to test for possible association. Fishers Exact Test and Chi square tests for significance were used. The study had been set within a 95% confidence interval and the level of significance at 0.05. The variables that showed possible association between their occurrence and infection with *L. pneumophila* included exposure to air conditioners and a history of past/ concurrent respiratory illness (Table 2). Most of the respondents were exposed to air conditioners at their places of work.

Table 1
Summary of demographic, socio-economic and clinical characteristics of respondents (n= 120)

Variable	Frequency	(%)
Gender		
Female	47	39.2
Male	73	60.8
Age		
Mean	38.68	
Work area		
N/A	31	25.8
Outdoors	27	22.5
Indoors	62	51.7
Air conditioners		
Exposed	31	25.8
Unexposed	89	74.2
Residence		
Urban	85	70.8
Rural	35	29.2
Piped hot water		
Exposed	66	55
Unexposed	54	45
Alcohol		
Consumes	57	47.5
Stopped <1year prior	19	15.8
Never Consumed	44	36.7
Smoking		
Smokes	40	33.3
Stopped <5years prior	15	12.5
Never smoked	65	54.2
Admission period prior to sample collection		
1 day	41	34.2
2 days	39	32.5
3 days	40	33.3
HIV status		
Positive	75	62.5
Negative	43	35.8
Unknown	2	1.7
History of past/ concurrent respiratory illness (pneumonia, TB)		
Positive	70	58.3
Negative	50	41.7

Table 2
Occurrence of L. pneumophila and its association with risk factors

Variable	Calculated p-value
Air conditioners	0.003*
Piped hot water	0.546
Alcohol	0.4
Smoking	0.190
History of past/ concurrent respiratory illness	0.021*
ISS status	0.577
Gender	0.135
Age	0.492
Employment/ work area	0.069
Residence	0.124
Air conditioners and work area	0.001*
Alcohol and smoking	0.000*

(i) The frequencies of socio-demographic factors obtained in Table 1 above was cross-tabulated with the occurrence of *L. pneumophila* to test for possible association.
(ii) Fishers Exact Test and Chi-square tests for significance were used.
(iii) The study had been set within a 95% confidence interval. The level of significance was 0.05.
*- Indicates the variables that showed possible association between their occurrence and infection with *L. pneumophila.*

DISCUSSION

This study revealed that 9.2% of pneumonia patients admitted at the medical wards of Kenyatta National Hospital between March and June 2007 were infected with *L. pneumophila* serogroup 1. These findings correlate with those of a study done in South Africa (8) on atypical causes of community-acquired pneumonia in which 8.7% of pneumonia patients tested positive for *L. pneumophila.* A serological study conducted in Zambia (10) revealed that 10% of the pneumonia patients had been exposed to *L. pneumophila* although a further study failed to indicate an increase in antibody titre.

Studies done elsewhere have shown that some populations have an increased risk to developing severe *legionella* infections. Some of the risk factors for community-acquired and travel-associated legionellosis include: being a male, the elderly (>65years), cigarette smokers, history of heavy drinking, pulmonary related illnesses, immunosuppresion, and chronic debilitating illnesses e.g. haematological malignancies (11).

According to this study, 22.58% of the pneumonia patients who were exposed to air conditioners tested positive for *L. pneumophila.* A dirty air filter can harbour pollen, fungi and bacteria and allow microorganisms into the room, possibly triggering an asthma attack, irritation of the eyes, nose and throat - even flu like illness. Air conditioning systems have been documented as one of the man-made habitats of *legionellae*. In this study, 38% of those who work indoors are exposed to air conditioners. The study also showed statistical significance between exposure to air conditioners and infection (p= 0.003), as well as between those who work indoors and exposure to air conditioners (p= 0.001). Other studies have also shown that contact with contaminated aerosol systems (like infected air conditioning systems) is associated with pneumonia due to *legionellae* (12). Air conditioners in large buildings can pose a more serious threat because they use reservoirs of water that can harbour harmful bacteria. Air filters should be vacuumed periodically and washed with a disinfectant to prevent mildew. The filter should be left to dry completely before reinstalling, while disposable filters should be replaced at recommended intervals.

Hot water systems have also been shown to be man-made habitats of *L. pneumophila*. The bacteria are more easily detected from swab samples of biofilm than from flowing water, suggesting that the majority of *legionellae* are biofilm associated (13). Aerosolisation or aspiration of contaminated water is a major route of transmission. However there was no statistical significance in the number of pneumonia patients infected with *L. pneumophila* and exposure to piped hot water systems (p= 0.546). This could be explained by the fact that most patients who confirmed using hot water only did so at their places of work, for washing hands or teacups. Most of them did not have hot water systems at home for showering, which would probably be a major source of infection.

The study also showed statistical significance between a history of respiratory illness and infection with *L.pneumophila* (p= 0.021). History of respiratory illness in this study included a past history of pneumonia and tuberculosis. Fifty eight per cent of all respondents have a history of past or concurrent respiratory illness, and of these 14.2% were positive for *L. pneumophila* urinary antigen. Pulmonary related illnesses weaken the immune system thereby weakening the body's ability to fight of infection (14). Patients with defective immune systems are susceptible to *legionellae* infection, especially when the defect involves cell-mediated immunity. Patients who have the human immunodeficiency virus may be at risk for relapsing infections (15). In this study 61.7% of all respondents were HIV positive and of these 12% were positive for *L. pneumophila* urinary antigen.

Fourty seven per cent of all respondents were active consumers of alcohol and of these, 12.3% were positive for *L. pneumophila* urinary antigen. Studies have shown that excessive alcohol consumption can contribute to contraction of community-acquired pneumonia. The increased risk of suffering from pneumonia in alcoholic patients exists due to the fact that the activity of their immune system decreases. Alcohol acts as a sedative and can diminish the reflexes that trigger coughing and sneezing. It also interferes with the action of macrophages. A study conducted at Lousiana State University Health Sciences Centre involving mice showed that it suppresses an immune system protein- interleukin-17 but its effect in humans is yet to be confirmed (16). Although this study showed that there was no statistical significance in the number of pneumonia patients infected with *L. pneumophila* and consumption of alcohol (p= 0.4), the high percentage of a positive history of alcohol consumption among pneumonia patients in general is worth investigating increased risk. The amount of alcohol consumed may also be a factor in its effect on the immune response of an individual.

The study found that 33.3% of the pneumonia patients were exposed to cigarette smoking. Just like alcohol, smoking is also known to interfere with the pulmonary immune response. Other studies have shown that chronic exposure to cigarette smoke can result in injury to the airways and damage of the cilia (17). It alters the efficiency of their beating so that bacteria entering the trachea have an increased likelihood of entering the lungs. Although this study showed that there was no statistical significance in the number of pneumonia patients infected with *L. pneumophila* and history of smoking (p=0.190), smoking may predispose the individual to infection with other pneumonia causing pathogens. Just like in previous studies, this study has also revealed a statistical

significance between alcohol consumption and cigarette smoking (p= 0.000). The potential combined adverse effects of alcoholism and cigarette smoking on lung defenses against pathogen infection probably increases the risk of developing serious disease.

There was no statistical significance in the number of pneumonia patients infected with *L. pneumophila* and gender (p= 0.135). Studies have shown however that the male sex is at more risk of infection with *L. pneumophila* than their female counterparts (11). In this study out of the eleven patients who tested positive for the antigen nine were male and two were female. The reasons why the male have been found to be more susceptible is yet to be established, but it could be due to more exposure to environmental pollutants based on their lifestyle.

From this study, the investigators recommended that there is need for public health education on routine inspection and maintenance of air conditioners and hot water systems; habitats and possible sources of infection with *Legionella pneumophila* and socio-demographic factors increasing risk of contracting pneumonia. Exposure to air conditioners is a key predisposing factor to infection with *L. pneumophila* and this should raise the index of suspicion among clinicians as they obtain a patient's medical history.

There is need for a larger multi-centre study on the prevalence of infection by *L. pneumophila* in pneumonia patients (both community acquired and nosocomial), existence of co-infection and the antibiotic susceptibility of isolated organisms. There is also a need to carry out studies on other causes of atypical pneumonia to provide information on the local epidemiological picture.

In conclusion, the hypothesis set at the beginning of the study that there are cases of *legionella pneumophila* infection among pneumonia patients was proved. Among the patients recruited in the study, 9.2% tested positive for infection *legionella pneumophila* serogroup 1.

Exposure to air conditioners and a history of past or concurrent respiratory illness have been found to predispose one to infection with the bacteria. Most of those exposed to air conditioners are exposed at their places of work hence there is need for routine inspection and maintenance of such equipment .

REFERENCES

1. Fraser, D. W. 1984. Sources of legionellosis, pg.277-280. A. Balows, C. Thornsberry, J. C. Feeley, and W. Jakubowski (ed.), Proceedings of the Second International Symposium on Legionella. American Society for Microbiology, Washigton, D.C.
2. Marston, B. J., Plouffe, J. F., File, T. M., *et al*. Incidence of community acquired pneumonia requiring hospitalisation- results of a population based active surveillance study in Ohio. *Arch. Intern. Med.* 1997; **157:** 1709-1718.
3. Boswell, T. C. J., and Kudesia, G. Serologic cross-reaction between *Legionella pneumophila* and *Campylobacter spp* in the indirect fluorescent test. *Epidemio. Infect.* 1992; **109:** 291-295.
4. Mathias M., Jurgen H. and Christian L. Laboratory methods for the diagnosis of legionella infections. *J. Micro. Meth.* 1998; **33:** 57-79.
5. Angela D., Kashuba, M. and Charles H. B. Legionella urinary antigen testing: Potential impact on diagnosis and antibiotic therapy. *Diagn. Microbio. Infect. Dis*. 1996; **24:** 129-139.
6. Lever, on behalf of the European Working Group for Legionella Infections (EWGLI) and Joseph C A. Travel associated legionnaires disease in Europe in 2000 and 2001. *Euro. Surveill.* 2003; **8:** 104.
7. Fisher, A., Laing, J., Stoeckel, J. E., *et al.* Handbook for Family Planning Operations Research Design. 2nd edition. *Population Council, New York*.
8. Maartens, G., Lewis, S.J., de Goveia, C., *et al*. A typical bacteria are a common cause of community acquired pneumonia in hospitalised adults. *S. Afr. Med. J.* 1994; **84:** 678-682.
9. Binax, Inc. 10. Southgate Road, Scarborough, Maine 04074, USA. US 1-800-257-9525. Outside US 1-609-627-8000. Fax: 207-730-5710.
10. Rolfe, M. A study of legionnaries disease in Zambia. *Ann. Trop. Med. Parasitology.* 1986; **80:** 325-328.
11. Straus, W.L., Plouffe, J.F., File, T.M., *et al*. Risk factors for domestic acquisition of legionnaries disease. *Arch. Intern. Med. 1996;* **156:** 1685-1692.
12. Cohen J, Powderly WG. Infectious Diseases. 2nd ed. New York, NY: Elsevier, 2004.
13. Rogers, J. and Keevil, C. W. Immunogold and fluorescein immunolabelling of Legionella pneumophila within an aquatic biofilm visualised by using episcopic differential interference contrast microscopy. *Appl. Environ. Microbiol*. 1992; **58**: 2326-2330.
14. Schlossberg, D. and Bonoan, J. *Legionella* and immunosuppresion. *Semin. Respir. Infect.* 1998; **13:** 128-131.
15. Pedro-Botet, M.L., Sopena, N., Garcia-Cruz, A., *et al. Streptococcus pneumoniae* and *Legionella pneumophila* pneumonia in HIV-infected patients. *Scand. J. Infect. Dis*. 2007; **39:** 122-128.
16. Judd S., Zheng M., Ye, P., *et al*. Effect of alcohol consumption on host release of interleuikin-17 during pulmonary infection with *Klebsiella pneumoniae*. *Clin. Exper. Res*. 2001; **25:** 872-881.
17. Stanley, P. J., Wilson, R., Greenstone, M. A., *et al*. Effect of cigarette smoking on nasal mucocilliary clearance and ciliary beat frequency. *Thorax*. 1986; **41:** 519-523.