

Detection of Indoor and Outdoor Avian Antigen in Management of Bird-Related Hypersensitivity Pneumonitis

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ABSTRACT

Background: In the management of hypersensitivity pneumonitis (HP), antigen avoidance is crucial to prevent the progression of disease. Indirect and unrecognized exposure to the antigen may continue for a long time if persistence of the causative antigen is not recognized. To make a correct assessment of the patients' environment, we tried to establish the methods to detect indoor and outdoor avian antigens.

Methods: Sixteen patients with bird-related HP, 4 asymptomatic breeders, and 6 healthy controls were examined. We prepared anti-pigeon dropping extracts (PDE) polyclonal antibody from rabbits. Air samples and house dust samples were analyzed by an antigen-capture ELISA with signal amplification using catalyzed reporter deposition.

Results: In air samples, avian antigen could be detected in patients with HP (0.73 ± 0.53 ng/m³) and asymptomatic breeders (0.63 ± 0.23 ng/m³). In house dust samples, the amount of avian antigen was higher in patients with HP (2.4 ± 1.8 µg/g) and asymptomatic breeders (4.1 ± 2.3 µg/g) than in the controls (0.1 ± 0.2 µg/g).

Conclusions: Detection of indoor and outdoor avian antigen might contribute to the correct diagnosis and appropriate managements of bird-related HP.

KEY WORDS

antigen avoidance, avian antigen, ELISA, hypersensitivity pneumonitis, pigeon dropping extracts

INTRODUCTION

Bird fancier's lung (BFL) is a type of hypersensitivity pneumonitis (HP), which is induced by inhalation of avian antigens.^{1,2} Immunoglobulins (IgA or degraded IgA) and intestinal mucin in bird dropping and bloom (a keratinous particles derived from bird feathers) are assumed to be causative avian antigens in BFL.³ In management of BFL, early diagnosis and avoidance of further exposure to the causative antigens are crucial.⁴ The diagnosis of acute BFL is relatively straightforward since the clinical features are usually obvious. However, chronic BFL is sometimes difficult to diagnose because most patients have few acute episodes.⁵ Several reports suggested that even a low exposure to wild birds⁶⁻⁹ and unrecognized exposure to feather duvets and others¹⁰⁻¹⁴ might lead to HP. In ad-

dition, symptoms and signs of HP sometimes persist even after discontinuation of raising birds.¹⁰

Only a few studies have been reported on the environmental measurement of avian antigen in dust and air.¹⁵⁻¹⁷ Craig *et al.* reported that avian antigen could be detected in dust samples using an inhibition enzyme-linked immunoassay (ELISA) for prolonged periods of time after bird removal and environmental cleanup.¹⁵ Curtis *et al.* also reported that pigeon antigen could be found in dust and air samples from a pigeon-infected school by a direct competitive ELISA.¹⁶ However, these methods are not sensitive enough to measure a small amount of avian antigen.

This study was conducted to establish a method for measurement of a small amount of indoor and outdoor avian antigen by a catalyzed signal amplification system and to evaluate the clinical significance of this

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Authors' contributions: This study was designed by JK and NI. The clinical data was collected by YM and avian antigen was analyzed by JK and KT. The manuscript was critically revised and prepared by NI and YY.

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Table 1 Characteristics of patients with bird-related HP

Case	Sex	Age	B.I.	Type of HP	Antibody to avian Ag	Lymphocytic proliferation to avian Ag	Laboratory-controlled inhalation or environmental provocation
1	M	61	1100	chronic	+	-	+
2	M	72	1000	chronic	-	+	+
3	F	74	0	chronic	+	-	+
4	F	66	0	chronic	+	N.D.	+
5	F	64	0	chronic	+	+	+
6	M	62	400	chronic	+	-	+
7	M	79	180	chronic	+	+	+
8	F	80	0	chronic	+	-	+
9	F	65	560	chronic	+	-	+
10	F	52	0	chronic	+	+	+
11	F	61	0	chronic	+	+	+
12	F	61	760	chronic	+	+	+
13	F	51	0	chronic	+	+	+
14	M	58	600	acute	+	+	+
15	M	78	560	chronic	+	-	+
16	M	54	760	chronic	+	-	+

M, male; F, female; B.I., Brinkman index (the number of cigarettes per day multiplied by years); +, positive; -, negative; N.D., not done.

method in the management of bird-related HP.

METHODS

SUBJECTS

The environments of 16 patients with bird-related HP (15 chronic HP and one acute HP) were assessed by a site-visit and measurement of avian antigen (Table 1). Patients with chronic HP presented with an insidious type except in a patient with a recurrent type (Case 13).⁵ The diagnostic criteria for chronic bird-related HP included (i) a history of avian contact, (ii) antibodies and/or lymphocyte proliferation to avian antigen, (iii) reproduction of symptoms related to HP by an environmental provocation or a laboratory-controlled inhalation test, (iv) either evidence of pulmonary fibrosis on histopathological analysis or honeycombing on computed tomographic (CT) scans, (v) either progressive deterioration of a restrictive impairment on pulmonary function over one year or respiratory symptoms related to HP of 6 months duration.⁵ Samples from 4 asymptomatic breeders and 6 healthy controls were also examined. The study conformed to the declaration of Helsinki and was approved by the internal review board at our institution. Informed written consent was obtained for each subject.

IMMUNOLOGICAL EXAMINATIONS

Pigeon dropping extracts (PDE) were obtained according to the method by Tebo *et al.*¹⁸ Fresh pigeon droppings were collected from a pigeon loft in Tokyo and stirred with 20 volumes of PBS for 24 hours. The fraction was extensively dialyzed against distilled water and lyophilized. Specific antibodies in sera and

BAL fluids to PDE were measured by an ELISA as previously reported.¹⁹

To examine lymphocyte proliferation to avian antigen, peripheral lymphocytes and bronchoalveolar lymphocytes (2×10^5 cells) were cultured in quadruplicate with pigeon sera in 96-well plates for 5 days at 37°C. These cells were incubated with ³H-thymidine for 20 hours and their radioactivity was counted by a scintillation counter. The results were expressed as a stimulation index, mean counts in stimulated cells divided by those in unstimulated cells. Each assay was considered positive when the stimulation index was more than 1.8.¹⁹

The laboratory-controlled inhalation test was conducted as previously reported.²⁰ Briefly, patients inhaled 2 ml of PDE (340 µg/ml) through a hand nebulizer. Tests were considered as positive when patients fulfilled three or more of the following criteria: (i) increased radiologic abnormalities, (ii) an increase in alveolar-arterial PO₂ difference (A-aDO₂) by more than 10 mmHg and/or a decrease of carbon monoxide diffusion capacity (DLco) by more than 20%, (iii) a decrease in vital capacity (VC) by more than 15%, (iv) an increase in peripheral leukocyte count by more than 30%, (v) an increase in C-reactive protein (CRP) by more than 1.0 mg/dl, (vi) an increase in body temperature more than 1.0°C and/or the development of systemic manifestations including chills and general fatigue, (vii) development of respiratory symptoms (cough and dyspnea).

COLLECTION OF AIRBORNE DUST AND HOUSE DUST

Airborne dust was also collected by a high-volume air

sampler (High Volume Air Sampler, Model HV-500F, Sibata Scientific Technology Ltd., Tokyo, Japan) with filters (Fiberfilm Filters T60A20, Pall Corporation, New York, NY, USA) at an air flow of 0.8 m³/min. Sampling time ranged from one to 38 hours. The air sample was stored at -80°C until analysis.

House dust was collected by a domestic electronic vacuum cleaner. The dust was stored at -80°C until processed. Approximately one gram of the dust stirred with 40 ml of phosphate-buffered saline solution (PBS) for 24 hours at room temperature. After centrifugation at 10000 rpm, soluble antigen was filtered under sterile conditions at <0.45 µm (Millipore, Watford, UK).

PREPARATION OF POLYCLONAL ANTIBODY

Polyclonal antiserum to PDE was prepared according to the method by Coligan *et al.*²¹ Before immunization, 2 ml of serum was removed from a rabbit to use as a control preimmune fraction (pre-serum). On day 1, the rabbit was injected with a 1 : 1 mixture of PDE and Freund's Complete Adjuvant (Sigma Chemicals, St. Louis, MO, USA). On day 29 and day 58, the rabbit was given additional injection of mixture of PDE and Freund's incomplete adjuvant (Sigma Chemicals). The rabbit was then bled on day 68 and polyclonal antiserum (post-serum) was stored frozen at -80°C until used. The serum was purified by using an IgG purification kit (ImmunoPure [ProteinA] IgG Purification Kit, Pierce, Rockford, IL, USA) and conjugated with biotin (Biotin Labeling Kit-NH₂, Dojindo Laboratories, Kumamoto, Japan).

ANTIGEN CAPTURE ELISA WITH CATALYZED SIGNAL AMPLIFICATION SYSTEM

Antigen capture ELISA for avian antigen was constructed. One hundred µl of sodium carbonate buffer solution at pH9.6 containing 100 ng of polyclonal antiserum (pre- and post-serum) were coated on ELISA plates (Immulon 2HB Flat Bottom Microtiter Plates, Thermo Electron Inc., Milford, CT, USA) overnight at 4°C. The plates were washed three times with PBS with 0.05% Tween (PBST) and blocked for 1 hours with 1% bovine serum albumin and then washed three times with PBST. Then standard pigeon dropping extracts (concentration: 10⁻⁷ to 10⁻² mg/ml) and samples were added to the plates and incubated at 37°C for 3 hours. The plates were washed six times and then 100 ng of biotin-labeled post-antiserum were added and incubated at 37°C for 30 minutes. The plates were washed six times and then VECTASTAIN Elite ABC Kits (Vector Laboratories, Burlingame, CA, USA) were used according to the manufacturer's instructions. To amplify the signal generated by the enzyme horseradish peroxidase (HRP), the kit of ELAST ELISA Amplification System Tyramide (PerkinElmer Life And Analytical Science, Inc., Wellesley, MA, USA) was used according to the manufacturer's

instructions. After the amplification step, the plates were washed again six times with PBST and 100 µl of O-Phenylene Diamine (OPD) solution was added. After 30 minutes the reaction was terminated by adding 50 µl of 4N H₂SO₄ and the color developed was measured in an ELISA reader with 490 and 655 nm filters.

STATISTICS

Statistical analysis was performed with post-hoc test using Scheffe's F test. All expressed values are mean ± SEM unless stated otherwise. Statistical analyses were performed using a software package of Statcel 2 (OMS Publishing Inc., Saitama, Japan).

RESULTS

In the environment of an asymptomatic breeder who was breeding one pigeon in the veranda, avian antigen could be detected inside the room 2 meters away from the veranda. The amount of the antigen gradually increased depending upon the sampling time (Fig. 1).

We applied this method to a patient who was breeding about 200 racing pigeons in the loft (Case 14), which was located about 27 meters away from his house (Fig. 2). We collected air samples in the front of the pigeon loft and his house, and also obtained house dust samples inside his house. The amount of avian antigen in the air samples was 1.0 ng/m³ in front of the pigeon loft and 0.3 ng/m³ in front of his house. In the house dust sample, the amount of avian antigen was 3.0 µg/g (Fig. 2). These results supported the existence of avian antigen around his environment. After he quitted breeding pigeons, his disease became stable without progression.

We obtained air samples from 5 patients with HP (Table 2), 4 asymptomatic breeders, and 6 healthy controls without obvious avian contacts. Avian antigen was detected in patients with HP (0.73 ± 0.53 ng/m³) and asymptomatic breeders (0.63 ± 0.23 ng/m³). However, no avian antigen could be detected in controls (Fig. 3).

We obtained house dust samples from 15 patients with HP (Table 2), 4 asymptomatic breeders, and 6 healthy controls without obvious avian contacts. The amount of avian antigen was significantly higher in patients with HP (2.4 ± 1.8 µg/g) and asymptomatic breeders (4.1 ± 2.3 µg/g) than in the controls (0.1 ± 0.2 µg/g) (Fig. 4). This system using PDE seemed to be applicable to detect various types of avian antigen, because the presumed causative antigen originated from birds other than pigeons in 8 of 15 patients (Table 2).

Three patients with bird-related HP (Case 1, 9, 15) had moved to other places which seemed to be free of birds to avoid of avian antigen. The amount of avian antigen in house dust samples was decreased in all cases, though a minute amount of the antigen was

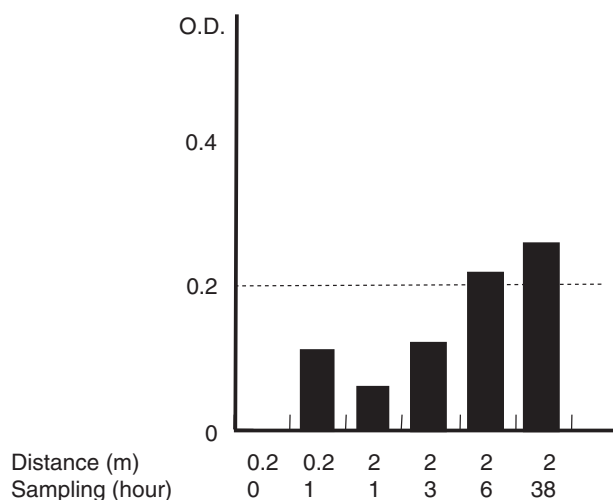


Fig. 1 Optical density (O.D.) in antigen capture ELISA for avian antigen in an asymptomatic pigeon breeder's room where one pigeon was bred in the veranda.

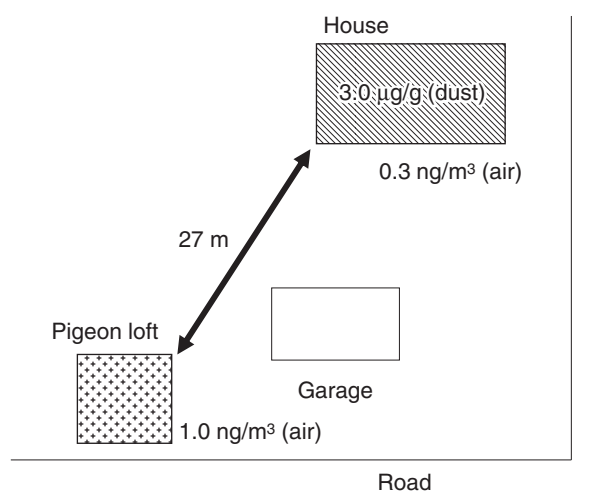


Fig. 2 A layout of a patient's yard with the amount of avian antigen. The patient was breeding about 200 pigeons 27 meters away from his house. Air samples were collected in front of the pigeon loft for one hour and in his house for 22 hours. Dust samples were obtained inside his house.

Table 2 Measurement of avian antigen in patients with bird-related HP

Case	Sampling place	Presumed causative antigen	Amount of avian Ag in 1m ³ of air (ng)	Amount of avian Ag in 1g of dust (µg)
1	House	200 racing pigeons 70 meters away from house	0.8	1.4
2	Workplace	A bird shop next door	1.6	1.2
3	House	Racing pigeons raised by a neighbor	N.D.	1.2
4	House	Breeding parakeets	N.D.	2.7
5	House	Pigeons raised by a neighbor	N.D.	0.8
6	Workplace	Birds in his workplace	N.D.	0.2
7	House	Many birds in neighbor's veranda	N.D.	3.0
8	House	Pigeons raised by a neighbor	N.D.	5.2
9	House	Use of poultry manure	N.D.	6.3
10	House	Wild birds in the yard	N.D.	1.1
11	House	Wild birds around house	0.6	4.0
12	House	Wild birds in the yard	N.D.	0.2
13	House	Pigeons in a railway station	N.D.	3.9
14	House	200 racing pigeons 27 meters away from house	0.3	3.0
15	House	Wild birds around house	N.D.	2.0
16	House	Breeding 10 pigeons	0.3	N.D.

Ag, antigen; N.D., not done.

still detectable in two cases (Fig. 5).

DISCUSSION

In the International Consensus Statements of ATS/ERS, chronic HP is one of the most important disease that must be differentiated from idiopathic pulmonary fibrosis (IPF) and nonspecific interstitial pneumonia (NSIP). We previously reported that 47% of patients with chronic BFL were initially misdiagnosed as having IPF or NSIP in our series.⁵ In management of patients with HP, antigen avoidance is crucial to prevent

the progression of disease. There are many obstacles to complete antigen avoidance in bird-related HP. First, it is relatively difficult to persuade patients to quit breeding birds because they are reluctant to understand the relationship between the occurrence of disease and bird breeding. Second, they have no chance to recognize the causative antigen when exposure to the antigen is indirect. In recent years, indirect and/or unrecognized exposures to birds including pigeons raised by neighbors, a flock of pigeons and/or wild birds in parks, shrines and railway sta-

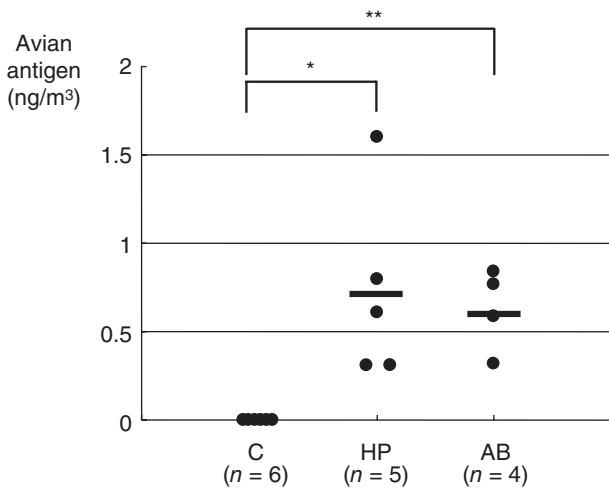


Fig. 3 The amount of avian antigen in air samples. Air samples were collected in front of each patient's house for 6 to 24 hours. C, control; HP, hypersensitivity pneumonitis; AB, asymptomatic breeder. Small bar: mean. * $p < 0.05$, ** $p < 0.01$.

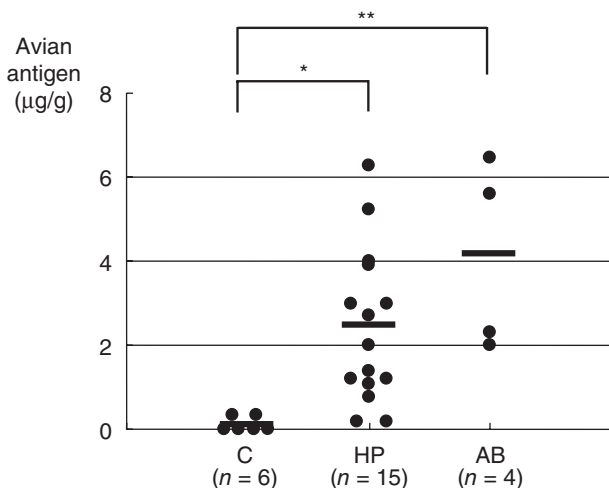


Fig. 4 The amount of avian antigen in house dust samples. C, control; HP, hypersensitivity pneumonitis; AB, asymptomatic breeder. Small bar: mean. * $p < 0.05$, ** $p < 0.01$.

tions are increasingly recognized to be locations responsible for HP.^{5,7,9,12} Third, there is no standardized method to clarify the existence of avian antigen in the patients' environment. Even if the patients try hard to avoid avian antigen, it is difficult to estimate whether avoidance is complete or incomplete.

Though a previous paper has reported a method to detect avian antigen using a direct competitive ELISA, the method is limited for detecting small amounts of avian antigen.¹⁶ To improve the sensitivity of detection of avian antigen, we employed a high volume air sampler to collect a large amount of airborne

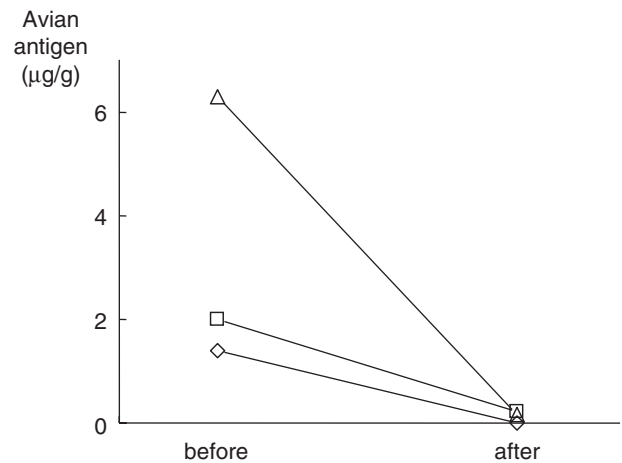


Fig. 5 The amount of avian antigen before and after antigen avoidance. Triangle: Case 9, square: Case 15, diamond: Case 1.

dust and applied an antigen capture ELISA with catalyzed signal amplification system. As a consequence, we could detect a small amount of avian antigen in dust and air samples from patients with bird-related HP and asymptomatic bird breeders. In the present study, we used anti-PDE polyclonal antibodies, because the exact epitope of the antigen responsible for BFL remains incompletely understood. In addition, antibodies against avian antigen are known to be cross reactive in various kinds of birds.³

Avian antigen was reported to persist in the patient's house 6 months after removal of all birds.¹⁵ Even after the patients with BFL quit breeding birds, existence of a minute amount of avian antigen might contribute to the progression of disease. To accomplish complete avoidance of the causative antigen, it should be important to evaluate a small amount of antigen in each patient's environment. In this study, a minute amount of avian antigen could be detected in the environments of 2 of 3 patients who moved to other places for antigen avoidance. These two patients seemed to slowly progress to an end-stage lung, whereas one patient without detectable avian antigen remained in stable condition. Relationships between the amount of avian antigen and the prognosis of bird-related HP should be further examined.

In conclusion, we were able to detect avian antigen in various environments. This method might be useful to prove existence of avian antigen in indoor and outdoor environments of patients with bird-related HP.

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REFERENCES

1. Fink JN. Hypersensitivity pneumonitis. *J Allergy Clin Immunol* 1984;**74**:1-10.
2. Kaltreider HB. Hypersensitivity pneumonitis. *West J Med* 1993;**159**:570-8.
3. McSharry C, Anderson K, Boyd G. A review of antigen diversity causing lung disease among pigeon breeders. *Clin Exp Allergy* 2000;**30**:1221-9.
4. Patel AM, Ryu JH, Reed CE. Hypersensitivity pneumonitis: current concepts and future questions. *J Allergy Clin Immunol* 2001;**108**:661-70.
5. Ohtani Y, Saiki S, Sumi Y *et al*. Clinical features of recurrent and insidious chronic bird fancier's lung. *Ann Allergy Asthma Immunol* 2003;**90**:604-10.
6. Kokkarinen JI, Tukiainen HO, Terho EO. Hypersensitivity pneumonitis due to native birds in a bird ringer. *Chest* 1994;**106**:1269-71.
7. du Marchie Sarvaas GJ, Merkus PJ, de Jongste JC. A family with extrinsic allergic alveolitis caused by wild city pigeons: A case report. *Pediatrics* 2000;**105**:E62-5.
8. Choy AC, Patterson R, Ray AH, Roberts M. Hypersensitivity pneumonitis in a raptor handler and a wild bird fancier. *Ann Allergy Asthma Immunol* 1995;**74**:437-41.
9. Saltoun CA, Harris KE, Mathisen TL, Patterson R. Hypersensitivity pneumonitis resulting from community exposure to Canada goose droppings: when an external environmental antigen becomes an indoor environmental antigen. *Ann Allergy Asthma Immunol* 2000;**84**:84-6.
10. Baverstock AM, White RJ. A hazard of Christmas: Bird fancier's lung and the christmas tree. *Respir Med* 2000;**94**:176.
11. Inase N, Ohtani Y, Endo J, Miyake S, Yoshizawa Y. Feather duvet lung. *Med Sci Monit* 2003;**9**:CS37-40.
12. Greinert U, Lepp U, Becker W. Bird keeper's lung without bird keeping. *Eur J Med Res* 2000;**27**:124.
13. Inase N, Ohtani Y, Sumi Y *et al*. A clinical study of hypersensitivity pneumonitis presumably caused by feather duvets. *Ann Allergy Asthma Immunol* 2006;**96**:98-104.
14. Meyer FJ, Bauer PC, Costabel U. Feather wreath lung: chasing a dead bird. *Eur Respir J* 1996;**9**:1323-4.
15. Craig TJ, Hershey J, Engler RJ, Davis W, Carpenter GB, Salata K. Bird antigen persistence in the home environment after removal of the bird. *Ann Allergy* 1992;**69**:510-2.
16. Curtis L, Lee BS, Cai D *et al*. Pigeon allergens in indoor environments: a preliminary study. *Allergy* 2002;**57**:627-31.
17. Edwards JH, Troman DM, Mason OF, Davies BH, Jones KP, Alzubaidy TS. Pigeon breeder's lung—the effect of loft litter materials on airborne particles and antigens. *Clin Exp Allergy* 1991;**21**:49-54.
18. Tebo TH, Moore VL, Noh JJ. Antigens in pigeon breeder's disease: isolation of a homogeneous antigen from pigeon dropping extract and its relationship to pigeon serum antigens. *J Reticuloendothel Soc* 1975;**18**:196-203.
19. Yoshizawa Y, Miyake S, Sumi Y, Hisauchi K, Sato T, Kurup VP. A follow-up study of pulmonary function tests, bronchoalveolar lavage cells, and humoral and cellular immunity in bird fancier's lung. *J Allergy Clin Immunol* 1995;**96**:122-9.
20. Ohtani Y, Kojima K, Sumi Y *et al*. Inhalation provocation tests in chronic bird fancier's lung. *Chest* 2000;**118**:1382-9.
21. Coligan JE. *Current Protocols in Immunology*. New York: John Wiley and Sons, 1997;1-9.