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Research paper

An immunoproteomic approach revealed antigenic proteins enhancing serodiagnosis performance of bird fancier's lung

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ABSTRACT

Background: Bird fancier's lung (BFL) caused by repeated inhalation of avian proteins is the most common form of hypersensitivity pneumonitis. However, the exact identification of proteins involved is unknown, and serological test use for diagnosis need to be standardized. The objectives of this study were (i) to identify antigenic proteins from pigeon droppings (ii) to provide information about their location in avian matrices and (iii) to produce them in recombinant proteins to evaluate their diagnostic performances.

Method: Antigenic proteins of pigeon dropping extracts were investigated using 2-dimensional immunoblotting with sera from patients with BFL, asymptomatic exposed controls and healthy volunteers. We investigated the origin of these antigenic proteins by analyzing droppings, blooms and sera using a shotgun proteomic analysis. BFL-associated proteins were produced as recombinant antigens in *E. coli* and were assessed in ELISA with sera from patients (n = 25) and subject exposed controls (n = 30). These diagnostic performances were compared with those obtained by precipitin techniques (agar gel double diffusion, immunoelectrophoresis).

Results: We identified 14 antigenic proteins mainly located in droppings and blooms. These proteins were involved in either the digestive or immune systems of pigeons.

Using the recombinant BFL-associated proteins: Immunoglobulin lambda-like polypeptide-1 (IGLL1: sensitivity: 76%; specificity: 100%; AUC: 0.93) and Proproteinase E (ProE: sensitivity: 84%; specificity: 80%; AUC: 0.85), the ELISA test showed better performance than precipitin assays with pigeon dropping extracts (sensitivity: 60%; specificity: 93.3%; AUC: 0.76).

Conclusion: IGLL1 and ProE were identified as the biomarkers of the disease. The use of these highly standardized antigens discriminates BFL cases from exposed subjects in serological assays. The results of this study offer new possibilities for the serological diagnosis of the disease.

Clinical trial registration: ClinicalTrials.gov: Identifier NCT03056404.

1. Introduction

Hypersensitivity pneumonitis (HP) is a group of inflammatory interstitial lung diseases caused by an exaggerated immune response to the inhalation of antigenic particles from environmental sources (Costabel et al., 2012). More than 300 agents have been reported to be involved in HP including bacteria, fungi, animal proteins and chemical compounds (Dalphin and Gondouin, 2015). These antigenic substances can be found in various locations including the home, recreational sites or the workplace. First described by Reed et al. in 1965 (Reed et al., 1965), bird fancier's lung (BFL) is the most common form of HP worldwide with a prevalence ranging from 6% to 20% of exposed

Abbreviations: BFL, bird fancier's lung; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; HP, hypersensitivity pneumonitis; AECs, asymptomatic exposed controls; DD, agar gel double diffusion; IEP, immunoelectrophoresis; HVs, Healthy volunteers; PDE, Pigeon Dropping Extract; 2-DE, 2 Dimensional-Electrophoresis; IGLL1, immunoglobulin lambda-like polypeptide 1; ProE, Proproteinase E

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pigeon breeders (Costabel et al., 2012).

Regular exposure to avian proteins from droppings, feathers and blooms (white powder that coats the feathers) may cause the disease in sensitized individuals. Following inhalation of antigens, immune complexes are formed in the alveolar and bronchiolar walls leading to tissue damage, fibrosis or emphysema. Evidence is supported by the presence of high titers of specific IgG antibodies to the offending antigens in both the circulation and bronchoalveolar lavage fluids (BAL) (Yoshizawa et al., 1995). The immunologic mechanisms involved in the disease have been described as a combination of type III (immune-complex-mediated reaction) and IV reaction (granuloma formation) with activation of alveolar macrophages and T lymphocytes (Costabel et al., 2012).

The diagnostic procedure of this pathology is complex and requires a combination of clinical, radiological, functional and biological criteria, including the finding of circulating antibodies against the offending antigen. For serological tests, several methods to determine precipitins (immunodiffusion and immunoelectrophoresis) (Grech et al., 2000; Funke and Fellrath, 2008; McSharry et al., 2006) or total IgG antibodies (enzyme-linked immunosorbent assays (ELISA) and ImmunoCAP technique) (Lopata et al., 2004) have been routinely used in analytical laboratories. Some of these techniques were very time-consuming to implement, and the antigens used often lacked standardization.

The efficacy of these tests can be improved by a standardized ELISA using recombinant antigens instead of crude extracts. To move forward in this field, the National Heart, Lung and Blood Institute/Office of Rare Diseases Workshop in Bethesda (Maryland) recommended the validation of biomarkers (exposure and disease) and the development of a battery of standardized antigens that should be available to clinicians for use in diagnosis (Fink et al., 2005). Indeed, there is a lack of knowledge about the precise identity of the antigenic proteins in BFL as well as their location in avian matrices. However, these data are very important both to better understand the disease and to provide breeders with targeted preventive measures. Novel emerging data in Genomics such as the whole genome sequencing of *Columba livia* (since September 2013) and in Proteomics (LC-MS/MS) now make it possible to characterize new candidate biomarkers for BFL.

Our strategy was to identify by LC-MS/MS all the antigenic proteins revealed by 2D-immunoblotting and also detected on the 2-DE Coomassie blue gel. These proteins were localized by analyzing pigeon droppings, blooms and sera using a shotgun proteomic approach. The antigenic proteins which reacted by immunoblotting with sera from all patients (7/7) and not with sera from control breeders (0/6) were defined as “BFL-associated”. These proteins were produced as recombinant antigens and their diagnostic performances were assessed by ELISA and compared with those of precipitin techniques.

2. Materials and methods

2.1. Study population

The protocol was approved by the local ethics committee (CPP-Est II 15/496). Patients with BFL (n = 25) were diagnosed in the Pneumology Unit at the University hospital of Besancon (France) and ULB Erasme Hospital of Brussels (Belgium). All patients were given a diagnosis according to the following criteria (Dalphin and Gondouin, 2015): (i) Exposure to offending antigens, (ii) Symptoms and HRCT compatible with HP and basal crepitant rales, (iii) BAL lymphocytosis, and (iv) Decreased DLCO during exercise. The clinical data of patients are shown in Table 1.

The control group consisted of 30 patients exposed to birds and classified as follows: 20 patients were diagnosed with other pulmonary diseases (interstitial lung disease (n = 13), pulmonary infection (n = 3), severe asthma (n = 3) and lung cancer (n = 1)) and 10 asymptomatic exposed controls (AECs) with no pulmonary disease.

Table 1
Clinical data of BFL patients.

	BFL patients (n = 25)
Mean age, yr (range)	65,5 (42–80)
Gender: male/female	10/15
Symptoms n (%): dyspnea; cough; basal crepitant rales	24 (96%); 20 (80%); 25 (100%)
BAL lymphocytosis (≥ 20%): data not obtained for 6 patients	17/19 (90%)
Radiologic data n (%): ground glass opacities; micronodular pattern; fibrosis	23 (92%); 20 (80%); 10 (40%)
Bird exposure	Pigeon (n = 14); budgerigar (n = 7), canaries (n = 2); poultry (n = 2)

Spirometry, a six-minute walk test, serology, a physical exam and standardized medical questionnaires were performed to rule out the diagnosis for AECs.

The serological screening test was carried out using agar gel double diffusion (DD) with a detection threshold of 2 precipitin arcs, and immunoelectrophoresis (IEP) was used to confirm the diagnosis with a positivity threshold of 3 precipitin arcs. The antigens used for these techniques correspond to the bird droppings to which breeders were exposed. Precipitin techniques were performed as previously described (Reboux et al., 2009) with sera from patients (n = 25) and controls exposed to birds but not BFL (n = 30).

Healthy volunteers (HVs; n = 5) were recruited during blood donation. A questionnaire was used to check that they were not exposed to a farming environment or birds.

2.2. Protein extract preparation

Droppings, blooms and sera were collected from pigeons in breeding farms located in the Franche-Comté region. The proteins contained in 1 g of pigeon droppings and blooms were extracted as described by Bestel-Corre et al. (2002). The pellet was suspended in 50 µL of 2-dimensional lysis buffer (Readyprep 2D Starter Kit Rehydration Sample Buffer, Bio-Rad, Hercules, CA, USA), and the protein concentrations were measured with the 2D Quant kit (GE Healthcare, Uppsala, Sweden) according to the supplier's instructions.

2.3. 2-DE gel, immunoblotting

Proteins of pigeon dropping extracts (PDE) (100 µg for 2-DE gels; 50 µg for immunoblot) were suspended in a 2-dimensional lysis buffer (Readyprep 2-D Rehydration/Sample Buffer, Bio-Rad). IEF using immobilized pH gradient strips (IPG strip, pH 4–7, 11 cm, Bio-Rad) was carried out on the Protean i12 IEF system (Bio-Rad), with a total focusing of 20,000 Vh. IPG strips were then equilibrated and loaded onto a Criterion TGX Stain-Free gel (Bio-Rad, USA) according to the manufacturer's manual. After electrophoresis, proteins were stained with Coomassie blue or blotted to a nitrocellulose membrane using the Trans-Blot Turbo transfer system (Bio-Rad) according to the supplier's recommendations. Immunoblotting was performed as previously described with a few changes (Rouzet et al., 2014). Sera and goat anti-human IgG alkaline phosphatase conjugates (Sigma-Aldrich, St Louis, MO, USA) were diluted 1:10,000 and 1:4000 respectively in TBS-Tween-BSA. The sera used were those of 7 BFL patients and 6 AECs exclusively exposed to pigeons and 5 HVs. All image acquisition was carried out with a GS-800 calibrated densitometer (Molecular Imager GS-800 USB calibrated densitometer, Bio-Rad). The intensity of the antigenic spots on membranes was evaluated using PDQuest 2D software (Bio-Rad). A BFL-associated spot was defined as only detected by antibodies of the 7 BFL patients and not from the sera of the 6 AECs. Spots detected by both groups were associated with exposure to