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1 **Bird fancier's lung serodiagnosis by automated r-IgLL1 ELISA**

2

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5

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12 **Running head:**

13 Bird Fancier's Lung serodiagnosis

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18

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23 used in this study.

24 **Highlights**

25

26 • Precipitin-based techniques are time consuming.

27 • Antigens extracted from bulk products lack standardization.

28 • The r-IgLL1 ELISA combined with immunodiffusion shows a sensitivity/specificity  
29 of 89%/93%.

30 **Abstract**

31 *Context:* Bird fancier's lung (BFL) is the most prevalent form of hypersensitivity pneumonitis  
32 (HP) worldwide. The current techniques used for the serological diagnosis of BFL all use  
33 crude extracts from feathers, droppings, and blooms as test antigens, which is associated with  
34 a lack of standardization and variability of the results. An antigenic protein, immunoglobulin  
35 lambda-like polypeptide-1 (IgLL1), isolated from pigeon droppings, was recently identified to  
36 be associated with BFL. We used genetic engineering to produce IgLL1 as a recombinant  
37 antigen.

38 *Aim:* We aimed to prospectively validate the use of an automated ELISA based on  
39 recombinant IgLL1 protein (r-IgLL1) as the test antigen for the serological diagnosis of BFL.

40 *Methods:* Immunoprecipitation (IP) techniques (immunodiffusion (ID),  
41 immunoelectrophoresis (IEP)) and ELISA using r-IgLL1 were performed concomitantly over  
42 10 months on 634 sera from patients with a BFL serodiagnosis request. Questionnaires were  
43 sent to obtain details on the avian exposure, clinical data, and final diagnosis. Concordance,  
44 sensitivity (Se), and specificity (Sp) of the two techniques were compared.

45 *Results:* In total, 72 completed questionnaires were returned with 18 cases of BFL diagnosed  
46 and 54 of non-BFL. The concordance between the ELISA and ID+IEP precipitation  
47 techniques was 71%. The combination of immunoprecipitation techniques showed a Se of  
48 78% and a Sp of 67%. The ELISA using r-IgLL1 showed a Se of 89% and a Sp of 91%. The  
49 automated r-IgLL1 ELISA test is sufficiently efficient to be used alone for the diagnosis of  
50 patients exposed solely to Columbidae.

51 In cases of other avian exposure, the Se and Sp of the r-IgLL1 ELISA used for screening  
52 combined with the immunodiffusion test for confirmation were 89% and 93%, respectively.

53 *Conclusions:* The automated ELISA using r-IgLL1 is a promising tool for BFL serodiagnosis.  
54 Replacing immunodiffusion by the automated ELISA using r-IgLL1 as a screening technique  
55 will be the basis of our future strategy for BFL serodiagnosis.

56

57 **Keywords:** automated ELISA, bird fancier's lung, serological diagnosis, IgLL1 recombinant  
58 antigen, precipitins

59 **1. Introduction**

60 Bird fancier's lung (BFL) is the most prevalent form of hypersensitivity pneumonitis (HP)  
61 worldwide.<sup>1</sup> Exposure may occur in occupational, recreational, or home settings following  
62 inhalation of avian proteins located in droppings, blooms, or feathers. The diagnosis combines  
63 clinical, radiological, and biological evidence.<sup>2</sup> The measurement of serum-specific  
64 immunoglobulin (SS-IgG) is proposed as a diagnostic test for all HP forms.<sup>3</sup> Two principal  
65 types of serological assays are available for the diagnosis of BFL. The first consists of  
66 immunoprecipitation (IP) methods, such as electrosyneresis (ES), immunodiffusion (ID), and  
67 immunoelectrophoresis (IEP), which evaluate the presence and the number of antigen-  
68 antibody complexes formed as precipitin arcs, which are numbered by manual reading; these  
69 IP techniques are qualitative and time-consuming.<sup>4</sup> The second consists of enzyme-linked  
70 immunosorbent assays (ELISAs), including a commercial kit (ImmunoCAP<sup>®</sup>, Uppsala,  
71 Sweden), which deliver a quantitative result (optical density OD) and are automatable.<sup>5</sup>  
72 ELISAs offer several advantages over other serological assays, including a reduced workload,  
73 higher throughput, shorter turnaround time, and lower cost. Common to all these techniques is  
74 the fact that the test antigens used are extracted from a bulk product (feathers, droppings,  
75 blooms) and, lack standardization.<sup>3,4</sup> Recently, the protein immunoglobulin lambda-like  
76 polypeptide-1 (IgLL1), isolated from pigeon droppings, was shown to be associated with  
77 BFL.<sup>6,7</sup> We previously produced this protein as a recombinant antigen and retrospectively  
78 assessed its diagnostic performance by a manual ELISA.<sup>6</sup> The use of recombinant antigens is  
79 recognized to be the best way to avoid variability between batches and laboratories.<sup>3,4</sup>

80 In our mycology department (University Hospital Besancon, France), the serological  
81 diagnosis of BFL has been based solely on IP techniques using crude antigens from bird  
82 droppings.

83 Here, we aimed to prospectively validate an automated ELISA using the recombinant protein  
84 IgLL1 (r-IgLL1) as the test antigen for the serological diagnosis of BFL in our routine  
85 workflow.

## 86 **Materials and Methods**

### 87 **2.1 Study design**

88 From September 2018 to June 2019 (10 months), serology for BFL was performed on 634  
89 samples by our mycology department (University Hospital Besancon, France).

90 In the 6 months following the serological analyses (January 2020), of the returned  
91 questionnaires, only 72/634 questionnaires included all the information presented in Table 1.

92 The classification was based on the response of the clinicians to the questionnaires according  
93 to the criteria defined by Vasakova et al. <sup>8</sup> Based on the received information, the 72 files  
94 were separated between 18 BFL and 54 Non-BFL avian exposed patients.

95 At the time of testing, patients had symptoms compatible with BFL and avian exposure. The  
96 tests were performed as a first step before the implementation of eviction measures. There  
97 were no recurrent patients in this study. Non-BFL patients were exposed to birds, but the final  
98 diagnosis retained by the clinicians was a lung disease different from BFL.

99 IP techniques (immunodiffusion (ID) and immunoelectrophoresis (IEP)) and the automated  
100 ELISA using r-IgLL1 were performed concomitantly (in the same week).

101 When blood samples were sufficient, 10 µL of the sera were used to evaluate the assay. No  
102 informed consent was required, because the serum analyses were performed in support of the  
103 clinical diagnosis of hypersensitivity pneumonitis, as permitted by requirements in France.  
104 Moreover, the result of this test was mentioned as an indication on the patient's report.

105

### 106 **2.2 Questionnaires**

107 Within three months of reception of the BFL serology request, questionnaires were sent to the  
108 prescribers; the questionnaires (Appendix 1) included questions to clarify the nature of avian



109 exposure of the patient, as well as questions on their clinical aspects. These questionnaires  
110 aimed to assess whether BFL was retained as the final diagnosis by the clinician and the  
111 performance of the test according to the type of avian exposure.

### 112 **2.3 Immunoprecipitation techniques**

113 A panel of five home-made crude antigens was used for the routine serological diagnosis of  
114 BFL (pigeon, budgerigar, canary, hen, and duck), as previously described.<sup>4,9</sup> We used ID as a  
115 screening method and IEP as a confirmation method. Each test antigen yielding more than  
116 two immunoprecipitation arc (three arcs for hen) by ID triggered a confirmatory test using  
117 IEP, for which two immunoprecipitation arcs was considered positive. Three levels of  
118 interpretation were possible: “absence of antibodies”, “moderate level of antibodies”, or  
119 “antibody level in favor of BFL”.

120

### 121 **2.4 Production of recombinant protein IgLL1**

122 Genetic engineering in E.coli-based cell-free protein expression system was used to produce  
123 r-IgLL1 as previously described.<sup>6</sup> In order to reduce inter-batch variability, each production  
124 was made from the same bacterial clone stored at - 80°C. The quality of the extract was  
125 visualized in Sodium dodecyl sulfate polyacrylamide gel electrophoresis. Each new batch was  
126 validated by an ELISA test using a defined cohort of sera from BFL patients and  
127 asymptomatic exposed controls. This test allowed to validate the ELISA conditions and to  
128 validate the threshold OD value by making a ROC curve (R software).

129

## 130 **2.5 Automated indirect ELISA using r-IgLL1**

131 The wells of 96-well plates (PolySorp Immunomodule<sup>®</sup>, Nalge Nunc Rochester, NY, USA)  
132 were coated by incubation with 100 µL of 5 µg/mL of r-IgLL1 in PBS (Sigma-Aldrich, Saint-  
133 Louis, USA) at 4 °C overnight. ELISA plates were blocked with 200 µL of 50 mmol/L  
134 NaH<sub>2</sub>PO<sub>4</sub>, containing 0.5% bovine serum albumin (BSA, Sigma-Aldrich, Saint-Louis, USA)  
135 and 60 g/L Sorbitol for 1 h at 37°C. Sera samples were diluted at 1/100 in a dilution buffer  
136 (PBS, pH 7.4, 0.1% BSA, 0.1% Tween 20).

137 All the following steps of this assay were run on the EVOLIS Twin Plus automate (Bio-Rad  
138 Laboratories, Hercules, CA). It is an open, automated microplate processor that allows easy  
139 creation of serological test protocols using an enzyme immunoassay format. Maintenance is  
140 performed weekly and monthly, as recommended by the supplier. In addition, a revision is  
141 carried out every year by the supplier in order to check the performances of the automaton.

142 The quality control samples that were tested in each run included: 1) a negative control  
143 (anonymous blood donor serum), and polyclonal rabbit antibodies (anti-IgLL1, RD-Biotech,  
144 Besancon, France) used as 2) a positive control (1.8 µg/mL) and as 3) a reference sample (0.4  
145 µg/mL). A volume of 100 µL of diluted sera was deposited in duplicate in the wells and  
146 incubated for 1 h at 37°C in the incubation chamber. After washing, plates were incubated for  
147 1 h at 37 °C with 100 µL of protein A peroxidase conjugates (CliniSciences, Nanterre,  
148 France) diluted at 1/4000. Plates were then washed (4 times) and incubated with 100 µL of  
149 3,3',5,5'-tetramethylbenzidine solution (TMB, SctyTek laboratories) for 10 min at room  
150 temperature. This reaction was stopped with H<sub>2</sub>SO<sub>4</sub> and the optical density (450nm/620nm) in  
151 the microwells was measured by the instrument spectrophotometer.

152 Samples were analyzed in duplicate and the two optical density values (OD) blank-corrected  
153 and averaged to calculate the standard deviation (SD) and coefficient of variation (CV). If the  
154 CV was > 20%, the analysis was repeated. An index was calculated as follows: index = mean  
155 of blank corrected OD replicates/mean of blank corrected OD reference sample. Three levels  
156 of interpretation were possible according to the value of the index: "*negative*" for values <  
157 0.8, "*intermediate*" for values between 0.8 and 1 (uncertainly zone), or "*positive*"  
158 for values > 1.

159

## 160 **2.6 Statistical analysis**

161 All statistical analyses were performed using R 4.1.0 software. The diagnostic performance of  
162 the ELISA was assessed by analyzing the receiver operating characteristic (ROC) curves  
163 (pROC package). Performance was evaluated using the area under the curve (AUC),  
164 sensitivity (Se), and specificity (Sp). The threshold that best discriminated between patients  
165 and controls was determined using the best combination of Se and Sp. The results were  
166 considered consistent in the following two cases: (1) the interpretation of the IP techniques  
167 was "*Absence*" and that of the ELISA test "*Negative*" and (2) the interpretation of the IP  
168 techniques was "*Moderate or Positive*" and that of the r-IgLL1 ELISA results "*Intermediate or*  
169 *Positive*".

170 Demographic and clinical data were compared between groups. The Chi-square test was used  
171 to compare categorical variables. Comparisons of continuous variables were performed using  
172 the non-parametric Kruskal-Wallis (K) test. The Wilcoxon signed rank test was used to  
173 compare r-IgLL1 index values between BFL (n = 18) and non-BFL samples (n = 54). *P-*  
174 *values* < 0.05 were considered statistically significant.

## 175        **3 Results**

### 176    **3.1 Clinical features**

177    The demographic and clinical data are presented in Table 1. The mean age was 68.4 years for  
178    the 18 BFL patients (11 women, 7 men) and 68 for the 54 non-BFL patients (22 women, 32  
179    men). Sixty-seven per cent of the BFL patients were non-smokers versus 48% of the non-BFL  
180    patients. There were no significant differences in the sex ratio, mean age, or smoking habits  
181    between the BFL and Non BFL-groups. Exposure to Columbidae (pigeons, doves, turtle  
182    doves) accounted for 63% of all patients (45/72). Among the 18 BFL patients, eight were  
183    exposed solely to Columbidae, two to poultry, one to canaries, and two to budgerigars. Five  
184    patients were exposed to several bird species. There were no significant differences in the  
185    distribution of avian exposure between the BFL and non-BFL patients.

186    The distribution of chronic and acute forms was respectively 44% and 56% for BFL patients.  
187    These 2 groups of patients had similar levels of anti-IgLL1 antibodies (Wilcoxon test, p-  
188    value: 0.92).

189    The antigenic eviction strategy was indicated in the questionnaire for 50% of BFL patients  
190    and was successful for 89% (8/9). All BFL patients presented a clinical and imaging pattern  
191    consistent with HP according to the criteria defined by Vasakova et al.<sup>8</sup> BFL patients  
192    presented a significantly higher percentage of lymphocytes in bronchoalveolar lavage (BAL)  
193    fluid (44.5%) than non-BFL patients (14%). The three most diagnosed conditions among non-  
194    BFL patients were diffuse interstitial lung disease (24%), idiopathic pulmonary fibrosis  
195    (22%), and asthma (13%). Six patients were diagnosed with another form of HP: four caused  
196    by regular exposure to mold (domestic [n = 2], agricultural [n = 2]), one caused by cardamom,  
197    and one due to regular exposure to rabbit litter.

	<b>BFL (n = 18)</b>	<b>Non-BFL (n = 54)</b>	<b>P</b>
Number of men (%)	7 (40%)	32 (59%)	0.219
Age (mean), years	68.4	68.0	0.939
<b>Tobacco, use, n (%)</b>			
Non-smoker	12 (67%)	26 (48%)	0.749
Past-smoker	3 (17%)	22 (41%)	
Active smoker	1 (6%)	2 (4%)	
Not mentioned	2 (11%)	4 (7%)	
<b>Exposure to birds, n (%)</b>			
Columbidae (Pigeon, Dove, Turtledove)	8 (44%)	37 (69%)	0.985
Poultry (Duck, Hen, Goose)	2 (11%)	7 (13%)	
Canary	1 (6%)	3 (5%)	
Budgerigar	2 (11%)	3 (5%)	
Multiple	5 (28%)	4 (8%)	
<b>HP classification</b>			
Chronic HP, n (%)	8 (44%)	NA	
Acute HP, n (%)	10 (56%)	NA	
<b>Antigenic eviction performed, n (%)</b>			
Yes	9 (50%)		
No	1 (6%)		
Not mentioned	8 (44%)		
Reduction of symptoms after eviction	8 (89%)		
<b>Symptoms consistent with HP</b> (Cough, Dyspnea, Crackles) n (%)	18 (100%)	30 (56%)	
<b>Analytical data</b>			
Number of BALs performed n (%)	12 (67%)	19 (35%)	
Lymphocytes %, median (P25-P75)	44.5 (18.5-54.75)	14 (4.5-23.5)	0.023*
<b>Imaging (HRCT) compatible with HP</b> (Ground-Glass opacities, Reticular, Honeycombing, centrilobular micronodules)	18 (100%)	12 (22%)	
<b>Other pathologies (n = 48)</b>			
Diffuse interstitial lung diseases (DILD)			
<i>DILD unspecified</i>		13 (24%)	
<i>IPF</i>		12 (22%)	
<i>Cardiac disease</i>		2 (4%)	
<i>Influenza</i>		3 (6%)	
<i>Tuberculosis</i>		1 (2%)	
<i>Lung cancer</i>		1 (2%)	
Asthma		7 (13%)	
COPD		4 (7%)	
Not mentioned		5 (9%)	
<b>Other HP disease (n = 6)</b>			
<i>HP domestic (mold)</i>		2 (4%)	
<i>Farmer's lung disease</i>		2 (4%)	
<i>HP caused by cardamom</i>		1 (2%)	
HP due to rabbit litter		1 (2%)	

198 **Table 1. Demographic and clinical data of 18 BFL and 54 non-BFL cases**

199 **3.2 Concordance between techniques and their performance**

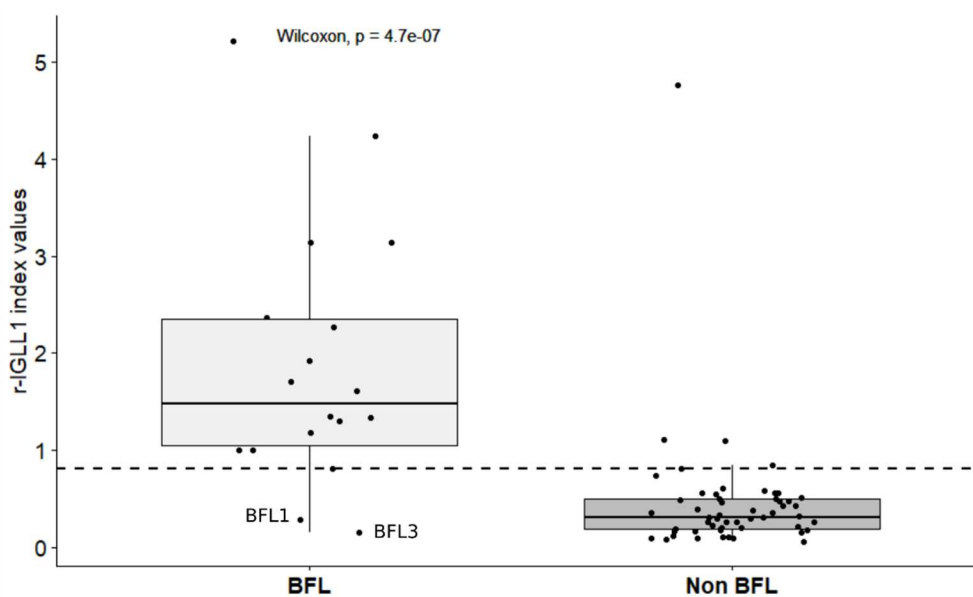
200 The antibody index r-IgLL1 values for BFL and Non-BFL patients are presented in Figure 1.

201 The antibody level appeared to be significantly higher for BFL patients than Non-BFL

202 patients for all avian exposure combined ( $p = 4.7e-07$ , Wilcoxon rank sum test). Based on

203 ROC analyses between BFL and non-BFL patients, the optimal threshold index of SS-IgG for

204 r-IgLL1 was 0.8, with a Se of 89%, a Sp of 91%, and an AUC of 0.9.



205

206 Figure 1. Boxplot of the index values for the r-IgLL1 ELISA for BFL (n = 18) and Non-BFL

207 patients (n = 54). The horizontal dotted lines represent the threshold determined by the ROC

208 analysis (highest AUC value) for r-IgLL1 (0.8). The Wilcoxon test indicated significantly

209 different index values between the BFL and non-BFL groups ( $p = 4.7 e-07$ ).

210

211 The results of the serological analyses (IP and ELISA) of the 18 BFL patients and five cases  
212 of Non-BFL patients classified as false positives by ELISA are presented in Table 2.  
213 The antigenic community was widely shared within the bird species (Table 2). Indeed, several  
214 patients (BFL6-BFL13; BFL17) exposed only to Columbidae showed sensitization to other  
215 species. Among the BFL patients 8/18 were solely exposed to Columbidae and the ELISA test  
216 was positive for all eight. Indeed, the ELISA test was “positive or intermediate” for 16/18  
217 BFL patients and negative for 49/54 non-BFL patients. Among the 18 BFL patients, the  
218 ELISA was negative in two cases (BFL 1, BFL 3). In the first case, patient BFL1 was exposed  
219 to several bird species (parrot, canary) and the results of the precipitin tests were also  
220 negative. For the second case (BFL3), the patient was exposed to budgerigars and the IEP  
221 results showed “moderate” sensitization to duck and hen antigens. The results of the ELISA  
222 test were positive for 100% of BFL patients exposed only to birds of the Columbidae family.  
223 The r-IgLL1 ELISA showed 5/54 false positives. Among them, 3/5 were exposed only to  
224 Columbidae and were also positive using precipitin tests. Patient “Non-BFL 2” was exposed  
225 to several bird species and patient “Non-BFL-5” to budgerigars. Neither patient showed  
226 sensitization by the precipitin-based techniques.

227 The interpretation of the results of the current precipitin techniques (ID followed by IEP) was  
228 in accordance with the clinician's diagnosis in 14/18 cases of BFL (Se = 78%). On the other  
229 hand, 36/54 non-BFL patients showed no sensitization, i.e., a test Sp of 67%.

230 The concordance between r-IgLL1 ELISA and IP-based techniques (ID and IEP) was 71%  
231 (51/72). The two techniques provided similar interpretations for 14/18 BFL patients and 37/54  
232 Non-BFL patients. We tested various combinations of techniques to choose the test that offers  
233 the best diagnostic accuracy. We obtained a Se of 72% and Sp of 94% using the ELISA test

234 for screening and IEP for confirmation, whereas the strategy using the ELISA test for  
235 screening and ID for confirmation resulted in a Se of 89% and Sp of 93%. The second  
236 combination would result in 30% (21/72) of serum samples being tested using the  
237 confirmatory technique (ID) instead of 92% (66/72) following the current strategy (ID  
238 followed by IEP).  
239



240 Table 2. Results of serological analysis (IEP, ELISA) for the 18 BFL patients and the five  
 241 cases of false-positive non-BFL patients. The symbol "-" was used to specify the absence of

ID	Exposure	r-IgLL1 index	Inter-ELISA	Inter-ID	New strategy ELISA + ID	Current Inter IEP	IEP results (number of arcs)					
							Pigeon	Budgerigar	Hen	Canary	Duck	Turtle-dove
<b>BFL1</b>	Parrot, Canary	0.28	Negative	Positive	Negative	Absence	0	-	-	-	1	-
<b>BFL2</b>	Poultry	1.91	Positive	Positive	Positive	Absence	0	-	1	0	-	-
<b>BFL3</b>	Budgerigar	0.16	Negative	Positive	Negative	Moderate	0	-	1	-	2	-
<b>BFL4</b>	Poultry	0.99	Intermediate	Positive	Positive	Absence	0	-	-	-	0	-
<b>BFL5</b>	Columbidae, poultry, swallows	1.29	Positive	Positive	Positive	Moderate	2	-	-	-	-	0
<b>BFL6</b>	Columbidae	3.14	Positive	Positive	Positive	Moderate	2	-	1	2	-	-
<b>BFL7</b>	Columbidae	1.70	Positive	Positive	Positive	In favor	2	3	-	-	-	-
<b>BFL8</b>	Columbidae	0.81	Intermediate	Positive	Positive	Moderate	2	-	1	-	0	-
<b>BFL9</b>	Columbidae	2.26	Positive	Positive	Positive	In favor	4	2	0	0	-	-
<b>BFL10</b>	Canary	1.00	Positive	Positive	Positive	Moderate	1	2	1	0	0	-
<b>BFL11</b>	Columbidae	1.60	Positive	Positive	Positive	In favor	3	3	3	2	0	-
<b>BFL12</b>	Columbidae	5.21	Positive	Positive	Positive	In favor	3	3	1	2	-	3
<b>BFL13</b>	Columbidae, Canary	1.33	Positive	Positive	Positive	In favor	3	2	-	-	-	-
<b>BFL14</b>	Columbidae, poultry	1.35	Positive	Positive	Positive	In favor	4	-	-	-	-	-
<b>BFL15</b>	Columbidae	3.14	Positive	Positive	Positive	In favor	1	-	-	-	-	3
<b>BFL16</b>	Columbidae, Poultry	2.36	Positive	Positive	Positive	Moderate	2	-	2	-	1	-
<b>BFL17</b>	Columbidae	4.24	Positive	Positive	Positive	Moderate	2	1	-	-	-	-
<b>BFL18</b>	Budgerigar	1.17	Positive	Positive	Positive	Absence	-	-	-	1	-	-
<b>Non-BFL 2</b>	Columbidae, Parrot, Budgerigar	0.85	Intermediate	Negative	Negative	Absence	-	-	-	-	-	-
<b>Non-BFL 3</b>	Columbidae	1.09	Positive	Positive	Positive	Moderate	2	-	-	-	1	-
<b>Non-BFL 5</b>	Budgerigar	4.76	Positive	Positive	Positive	Absence	0	-	-	-	-	-
<b>Non-BFL 14</b>	Columbidae	0.80	Intermediate	Positive	Positive	Moderate	2	-	-	1	0	-
<b>Non-BFL 20</b>	Columbidae	1.10	Positive	Positive	Positive	In favor	3	2	-	-	0	-

242 data (the analysis was not performed).

243 *Discussion*

244 Here, we evaluated the performance of the r-IgLL1 ELISA and compared it with precipitin  
245 assays (ID, IEP) routinely used in our laboratory.

246 The ELISA appears to be a powerful tool for the screening of BFL for serological diagnosis.  
247 Indeed, this assay showed a Se of 89% and a Sp of 91% for sera from patients exposed to  
248 birds and experiencing various pulmonary pathologies. Such performance was superior to that  
249 obtained using precipitin tests (Se of 78%, Sp of 67%). IP-based techniques, the techniques  
250 principally used for the serodiagnosis of HP, are qualitative (manual reading of  
251 immunoprecipitation arcs), time-consuming, and require the use of test antigens, consisting of  
252 extracts from bulk products (droppings, blooms, feathers, hay, etc.) or cultured  
253 microorganisms.<sup>3,4</sup> Moreover, the equipment and materials for such IP-based techniques are  
254 becoming increasingly more difficult to obtain (for example, the recent discontinuation of the  
255 marketing of cellulose acetate strips in France). In addition, the change of supplier of  
256 cellulose acetate strips require important adjustments to the protocol.

257 Another automated method for serodiagnosis of BFL was the immunoCAP. The catalog of  
258 commercial antigens is relatively extensive (n=15 antigens). However, the supplier did not  
259 provide threshold values for positivity and studies with this technique have shown  
260 significantly different thresholds for the same antigen. For pigeon antigen, the optimal cutoff  
261 value was determined to be 9.8 mgA/L by Lopata *et al.*<sup>10</sup> and 24.6 mgA/L by Shirai *et al.*<sup>11</sup>  
262 Performances also showed variations between studies depending on the patient cohorts but  
263 may also be related to the nature of the antigen. The immunoCAP antigen were crude antigens  
264 for which it seems difficult to guarantee long-term standardization. Indeed, the proteins

265 localized in the droppings of the birds can vary according to the metabolism of each pigeon,  
266 according to the diet or according to the stress undergone by the bird.

267 The use of recombinant proteins as test antigens is recognized as the best way to avoid  
268 variability between batches and laboratories.<sup>3</sup> Such recombinant antigens can be used in  
269 automated ELISAs, which is a non-negligible time-saving.<sup>3,4</sup> In addition, ELISA tests offer  
270 the possibility to follow patient antibody levels according to the measures taken to improve  
271 their health (e.g. antigenic avoidance).

272 Immunoglobulin-lambda like-polypeptide 1 (IgLL1) and Proproteinase E (ProE) were  
273 characterized as a BFL-associated protein using an immunoproteomic approach.<sup>6</sup> These  
274 proteins were detected by antibodies of sera from 7/7 BFL patients and 0/6 sera from avian-  
275 exposed healthy controls. Both IgLL1 and ProE proteins were good candidates for  
276 serodiagnosis of BFL cases. Indeed, BFL patients have a higher level of antibodies against r-  
277 IgLL1 than against r-ProE.<sup>11</sup> Furthermore, we demonstrated that unlike r-IgLL1, r-ProE was  
278 effective for the serodiagnosis of feather duvet lung (FDL) (Se: 74.2%, Sp:86.7%, AUC:  
279 0.89).<sup>11</sup> Therefore, the antigens r-ProE and r-IgLL1 could be used respectively for the  
280 serological diagnosis of patients exposed strictly to feathers bedding or birds and presenting  
281 with respiratory symptoms.

282 Shirai *et al*, showed that r-IgLL1 provoked a BFL-specific response in ELISA.<sup>7</sup> This study  
283 confirms these results in routine and the absolute novelty concerns the automation of this  
284 device which offers a saving of time and a better standardization of the results. IgLL1 was  
285 first identified from pigeon droppings and then produced from the amino acid sequence of this  
286 species.<sup>6,7</sup> However, it has been shown that IgLL1 is highly conserved between bird species,  
287 participating in antigenic cross-reaction.<sup>6-8</sup> The r-IgLL1 ELISA is efficient for the serological

288 diagnosis of the main sources of avian exposure: Columbidae (pigeon, dove, turtledove),  
289 budgerigars, canary and poultry. It is very likely that this protein can be recognized by the  
290 antibodies of patients exposed to other avian species. However, it will be necessary to validate  
291 this test for other types of exposure to delimit the range of effectiveness of this antigen. It is  
292 crucial that the source of avian exposure be provided by the clinician on the request form to  
293 perform the serological analysis under the best conditions.

294 Based on the results of this study we believe that the use of the r-IgLL1 ELISA is sufficient  
295 when breeders are solely exposed to birds of the Columbidae family. However, in the event of  
296 multi-exposure or exposure to other species, it is useful to use the immunodiffusion test for  
297 confirmation. This combination was associated with a Se of 89% (16/18 BFL) and a Sp of  
298 93% (50/54 non-BFL). Intermediate and positive results of the ELISA using r-IgLL1 could be  
299 confirmed by ID using a panel of six house-made crude antigens (pigeons, budgerigar, canary,  
300 hen, duck, and parrot). Bird lovers collect atypical avian species, for which the efficiency of  
301 this test is not yet known. Thus, custom-made antigens (i.e., made from the droppings of the  
302 patient's bird) should be made if needed and tested by ID. In all cases, the final diagnosis is  
303 based on a combination of clinical, radiological, and biological evidence.

304 This prospective study provided the opportunity to incorporate the automated ELISA using r-  
305 IgLL1 in our routine workflow. Based on these results, we plan to replace ID by the  
306 automated r-IgLL1 ELISA as a screening technique. We will still use ID, but no longer as a  
307 screening technique but rather as a confirmatory test. This strategy is in accordance with the  
308 results of the study of Shiroshita et al.<sup>12</sup>, who demonstrated high sensitivity for the ELISA test  
309 and high specificity for the Ouchterlony test (other denomination of ID).

310

311 **4. Conclusions**

312 The automated r-IgLL1 ELISA responded favorably to the presence of antibodies for the  
313 diagnosis of 16/18 BFL patients. This test is therefore sufficient for the serodiagnosis of  
314 patients exposed to birds of the Columbidae family (63% in this study).

315 Furthermore, this serological test could be a relevant tool to measure the kinetics of the  
316 antibody level after the implementation of the antigenic avoidance strategy. The strategy (r-  
317 IgLL1 ELISA + ID) proposed in this study performed well (Se = 89%, Sp = 93%) in  
318 discriminating between bird-exposed patients with BFL and exposed patients with other lung  
319 pathologies. The continued use of an IP-based technique has the advantage of providing an "à  
320 la carte" panel of antigens according to the specific source of the patient's exposure. To be  
321 effective, this new approach requires precise information about the source of avian exposure  
322 of the patient. In addition, in the case of exposure to bedding feathers, the antigen chosen in  
323 ELISA will be r-ProE. Moreover, an automated ELISA using respectively r-IgLL1 for BFL  
324 and r-ProE for FDL serodiagnosis represents a timely alternative for laboratories confronted  
325 with the impossibility of further performing ES due to the discontinuation of the marketing of  
326 cellulose acetate strips

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