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TECHNICAL REPORT

Detection of serum immunoglobulins in wild birds by direct ELISA: a methodological study to validate the technique in different species using antichicken antibodies

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Summary

1. This study presents an easy protocol to measure the amount of immunoglobulins from the blood serum of different bird species in the wild (*Ficedula hypoleuca* Pallas, *Parus caeruleus* L., *Lanius meridionalis* Temminkck, *Lanius collurio* L., *Athene noctua* Scopoli and *Falco tinnunculus* L.) by direct enzyme-linked immunosorbent assay, ELISA using commercial antichicken antibodies.

2. Additionally, the ELISA technique is validated for detecting serum immunoglobulins by means of other electrophoretic (sodium dodecyl sulphate polyacrylamide gel electrophoresis, SDS-PAGE, and native electrophoresis) and immunological (Western blot) methods.

3. The results by Western blot show that the commercial antibody recognized proteins with apparent molecular weight according to heavy and light chains of immunoglobulins.

4. Both ELISA and Western blot data were correlated, implying that the commercial antibody bound to immunoglobulins and not to other proteins or ELISA plates. Densitometric data achieved by SDS-PAGE and native electrophoresis were only correlated in some species indicating a problem in detecting clearly the heavy and light chains, and γ-globulin fraction, respectively.

5. It is concluded that the proposed protocol is easy to carry out and may be used to detect total serum immunoglobulins from most bird species.

Key-words: Ecological immunology, electrophoresis, humoral immunity, Western blot

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Introduction

The immune system has recently attracted a great deal of attention in evolutionary ecology, starting from the observation that parasitism exerts a strong selective pressure on most aspects of organismic biology (Sheldon & Verhulst 1996; Zuk *et al*. 1996). The main focus, rooted in life-history theory, assumes fitness costs of immune defence against parasites and trade-offs in the face of limited resources (e.g. Deerenberg *et al*. 1997; Nordling *et al*. 1998; Moreno, Sanz & Arriero 1999; Merino, Møller & de Lope 2000; Soler *et al*. 2002). Measurements of immune response are becoming routine in many ecological studies (Norris & Evans 2000), requiring the use of simple standardized techniques for estimating levels of activity of different components of the immune system.

The immune response has been differentiated into innate, cell-mediated and humoral components, although the operation of the immune defence is arguably one of the most complex phenomena in biology. Measurement of the cell-mediated response against different antigens, mainly phytohaemagglutinin, has become a standard technique in ecological studies (Merino *et al*. 1999; Smits, Bortolotti & Tella 1999, 2001; Moreno *et al*. 2001). On the other hand, the humoral response can be measured as an increase in levels of total antibodies (e.g. Johnsen & Zuk 1999; Szép & Møller 1999) or of specific antibodies against a non-pathogenic antigen such as diphtheria–tetanus vaccine (e.g. Ilmonen, Taarna & Hasselquist 2000; Råberg *et al*. 2000) or sheep red blood cells (Deerenberg *et al*. 1997; Saino, Calza & Møller 1997).

Initially, the quantification of antibodies from wild birds was made by means of native electrophoresis determining the γ-globulin fraction in serum (Gustafsson

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et al. 1994; Saino & Møller 1996; de Lope, Møller & de la Cruz 1998; Christe *et al*. 2001; Hoi-Leitner *et al*. 2001), but recently researchers have used immunological techniques such as agglutination (Cichon, Dubiec & Chadzinska 2001; Møller *et al*. 2001) and enzyme-linked immunosorbent assay, ELISA (Nordling *et al*. 1998; Svensson *et al*. 1998; Fair, Hansen & Ricklefs 1999; Hasselquist *et al*. 1999; Johnsen & Zuk 1999). At present, the ELISA technique is increasingly used by researchers to detect specific immunoglobulins from wild birds (Fair & Myers 2002; Gasparini *et al*. 2001, 2002; Ilmonen, Taarna & Hasselquist 2002). However, no study to date has tried to estimate total immunoglobulin concentrations in blood without antigen administration except in fowl (e.g. Johnsen & Zuk 1999). Estimating the total concentration of immunoglobulins does not require prior capture for immunization and may give a valuable insight into the health or nutritional state of birds in natural conditions (Gustafsson *et al*. 1994). The interpretation of relative levels of serum immunoglobulins regarding health status and immunocompetence in wild animals remains contentious and requires a large comparative data base including populations of many species under different environmental conditions. Here, we aim only at validating an easy ELISA protocol which may aid researchers in collecting relevant data which can aid in such an interpretation.

The main problem with using ELISA for estimating total serum immunoglobulin concentration in wild birds is the lack of commercial antibodies for detecting immunoglobulins from species other than the domestic chicken or anti-wild bird antibodies recently generated using sera of four species (Bethyl Laboratories, Montgomery, TX, USA; Ebel *et al*. 2002). There are two solutions for solving this problem: (i) to develop a specific antibody against the immunoglubulins from the species of interest (Hasselquist *et al*. 1999; Smits & Bortolotti 2001) or (ii) to use an antibody developed for other species such as domestic chicken (Phalen, Wilson & Graham 1995; Svensson *et al*. 1998), *Agelaius phoeniceus* (Råberg *et al*. 2000; Ilmonen *et al*. 2000, 2002) or using the antiwild bird immunoglobulins (Ebel *et al*. 2002). The first solution is laborious and economically costly. The second solution may be more practical because immunoglobulins in birds are encoded by a limited number of genes and sequences are more conserved than in other organisms (Roitt, Brostoff & Male 1993), although potential cross-reactivities against immunoglobulins in general and IgG in particular require validation. Thus, using commercial antibodies should be feasible, although the following considerations should be kept in mind: (a) What serum dilution should be used? (b) Are the anti-immunoglobulins binding to the right protein?

In this study we present an easy protocol to detect total immunoglobulins by direct ELISA from some wild bird species using a commercial antichicken antibody. We selected this antibody because of its ease of acquisition through several commercial companies. Additionally, we validate the ELISA technique for detecting immunoglobulins by means of other electrophoretic (sodium dodecyl sulphate polyacrylamide gel electrophoresis, SDS-PAGE, and native electrophoresis) and immunological (Western blot) methods. By using Western blot, we detect those proteins that are recognized by the antibody used (antichicken) and we estimate their molecular weight. Thus, we can confirm if the secondary antibody binds to the light and heavy chains of immunoglobulins or cross-reacts with some other serum protein. A positive correlation between the data obtained by ELISA and those obtained with Western blot would be the best control to validate that the bond produced in the ELISA plates is a specific one. On the other hand, the correlation of the data from the γ-globulin fraction obtained with native electrophoresis with data from ELISA would ensure that the antibody is not binding non-specifically to the albumin of the sera. This control is important as albumin has a similar molecular weight to the heavy chains of immunoglobulins. Another way of controlling for the bond with albumin would be to correlate the ELISA data with those obtained with Western blot only for the light chains. Given that one molecule of IgG has two heavy and two light chains, the immunoreactivities of the same number of heavy and light chains should be positively correlated. Finally, the use of SDS-PAGE in reduced conditions would simply validate the ELISA method with another electrophoretic technique.

Materials and methods

SERUM AND ANTIBODIES

We used sera from 12 wild individuals from each of the following bird species: Pied Flycatcher *Ficedula hypoleuca* Pallas (adults), Blue Tit *Parus caeruleus* L. (adults and nestlings), Grey Shrike *Lanius meridionalis* Temminck (adults), Red-Backed Shrike *Lanius collurio* L. (adults), Little Owl *Athene noctua* Scopoli (adults) and European Kestrel *Falco tinnunculus* L. (adults and nestlings). The antibody to detect immunoglobulins from the above species was a polyclonal rabbit anti-chicken IgG conjugated with peroxidase (Sigma A-9046, St Louis, MO, USA). This antibody recognizes the whole molecule.

SDS-PAGE

SDS-PAGE was carried out under denaturing conditions (2-β-mercaptoethanol) using a discontinuous buffer system (Laemmli 1970). Serum proteins (3 μl of serum at 1/10 dilution) were separated on polyacrylamide gels containing a stacking gel of 4% and a running gel of 10%. Electrophoretic buffer (25 mM Tris, 196 mM glycine and 0·1% SDS) and running conditions were according to recommendations of Bio-Rad (200 V; see Mini Protean III instructions) (Bio-Rad,

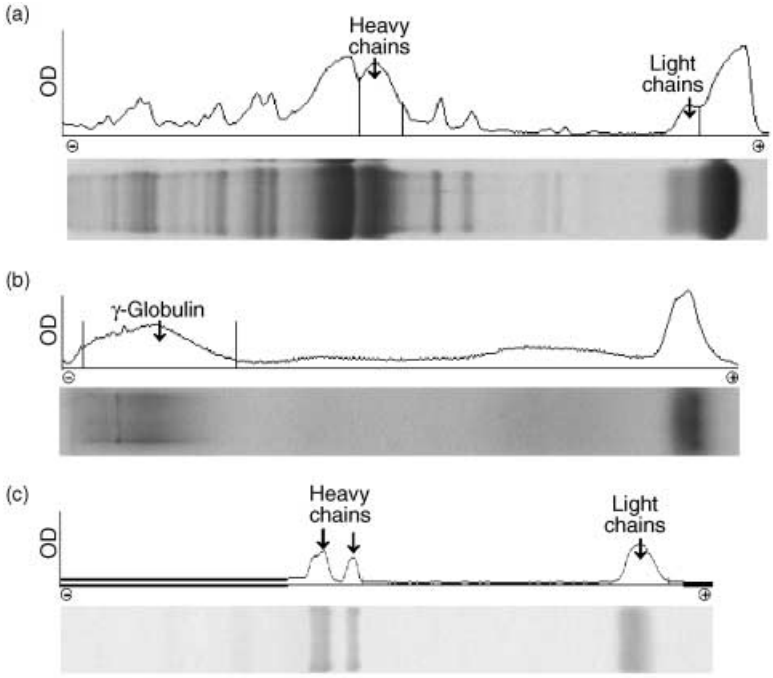

Fig. 1. (a) Example of an SDS-PAGE profile. (b) Example of a native electrophoresis profile. (c) Example of a Western blot profile.

Hercules, CA, USA). Once we had finished the electrophoresis, gels were covered with a staining solution (Coomassie Brilliant Blue R250 at 0·1% w/v in 40% methanol plus 10% acetic acid) for 1 h at room temperature on a shaker. Destaining of gels was made with 40% methanol plus 10% acetic acid on a shaker until a good contrast was obtained. Lastly, gels were scanned and protein bands quantified using 1D image analysis software (Scion Image for Windows, Scion Corporation, Frederick, MD, USA).

N-PAGE (NATIVE PAGE)

Native electrophoresis was carried out according to recommendations of Bio-Rad (see Mini Protean III instructions). Basically, the differences with SDS-PAGE were the following: (i) preparation of samples without 2-β-mercaptoethanol and SDS (ii) use of 6% running gels without stacking gel and (iii) electrophoretic buffer without SDS. The process to stain and analyse the polyacrylamide gels was the same as in the SDS-PAGE.

WESTERN BLOTTING

Serum proteins were prepared for SDS-PAGE under denaturing conditions. Proteins (3 µl of serum at 1/10 dilution) were separated on polyacrylamide gels containing a stacking gel of 4% and a running gel of 10% (Laemmli 1970) and transferred to Immobilon-P (polyvinylidene difluoride (PVDF) millipore) membranes (transfer buffer: 12·5 mM Tris, 98 mM glycine and 10% methanol; transfer conditions: 150 V for 1·5 h at 4 °C). Unstained markers for molecular weight from Sigma (references SDS-7 and SDS-6H) were included in all gels. After transference the PVDF membrane was stained with Red Ponceau 0·1% (diluted in 0·1% acetic acid) with the aim of separating the lanes containing the molecular weight markers. Then, transfer membranes without molecular weights were washed with 150 mM phosphate-buffered saline, pH 7·2 plus 0·05% Tween 20 (PBS-T) and incubated in blocking buffer (PBS-T plus 5% non-fat dry milk; Nestlé, Vevey, Switzerland). Later, blots were incubated with antichicken IgG conjugated with peroxidase (1/5000 dilution) diluted in PBS-T. Positive bands were detected by incubation with a developer solution (0·06% 3,3′-diaminobenzidine plus H_2O_2 at 1/1000 dilution). The incubation period for blocking buffer and antichicken antibodies was 1 h and 2 h at room temperature, respectively. Three washes (5 min) with PBS-T were performed after each step. Once the process was finished, blots were dried and the markers were once again bound to the membrane containing the immunoreactive bands in order to determine the apparent molecular weight of the bands. As the colour of the markers is usually very light, we painted the base of each marker before scanning the membrane. Later, we substituted the painted markers with lines. This procedure is nowadays a standard one. Lastly, blots were dried and immediately scanned. Protein bands were quantified using 1D image analysis software (Scion Image for Windows, Scion Corporation). Immunoreactivity was measured as optical density × area of the peaks (see Fig. 1c).

ELISA

ELISA plates (Maxi-sorp, Nunc, Rochester, NY, USA) were coated with serial dilutions of serum (100 µl) in carbonate–bicarbonate buffer (0·1 M, pH = 9·6, overnight at 4 °C) in order to determine the linear range of the sigmoid curve (Table 1). Later, the plates were blocked with defatted milk diluted in PBS-Tw buffer for 1 h at 37 °C (200 µl). Antichicken conjugate was added at 1/250 dilution in PBS-Tw and incubated for 2 h at 37 °C (100 µl). The dilution of antichicken antibody was selected after a previous study to achieve both the maximum slope in the linear range and the minimum cost. In addition, antichicken antibodies were diluted without any protein (i.e. BSA, gelatine, defatted milk, etc.) which avoids unspecific binding. The specificity for binding with immunoglobulins of antichicken antibodies was previously tested by Western blot. After incubation with a substrate comprising ABTS (2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)) and concentrated hydrogen peroxide diluted to 1/1000 for 1 h at 37 °C, absorbances were measured using a plate spectrophotometer at λ = 405 nm. In these conditions, we achieved the maximum values of absorbances.

Table 1. Total and linear range of absorbances and dilutions of serum

Species	Range of absorbances[b]	Linear range	Absorbances in the linear range
Ficedula hypoleuca	0·90 (0·05)–0·33 (0·02)	1/4000–1/32 000	1·00 (0·03)–0·46 (0·04)
Parus caeruleus	0·79 (0·24)–0·22 (0·04)	1/2000–1/8000	0·67 (0·22)–0·43 (0·13)
Parus caeruleus[a]	0·35 (0·12)–0·16 (0·02)	1/1000–1/4000	0·32 (0·09)–0·26 (0·08)
Lanius meridionalis	1·75 (0·45)–0·50 (0·29)	1/4000–1/64 000	1·43 (0·51)–0·50 (0·29)
Lanius collurio	1·28 (0·45)–0·35 (0·16)	1/4000–1/32 000	1·02 (0·46)–0·54 (0·29)
Athene noctua	1·35 (0·60)–0·35 (0·15)	1/4000–1/32 000	1·22 (0·52)–0·57 (0·30)
Falco tinnunculus	0·98 (0·28)–0·27 (0·05)	1/4000–1/32 000	0·84 (0·27)–0·35 (0·10)
Falco tinnunculus[a]	0·55 (0·10)–0·20 (0·02)	1/4000–1/32 000	0·46 (0·10)–0·20 (0·02)

[a]Nestlings.
[b]Range of absorbances at minimum dilution (1/500) and maximum dilution (1/64000). Data represent mean values ± standard deviation (deviation data in parentheses).

Table 2. Correlation (r^2) between immunoglobulin data achieved by four methods

Species	ELISA *vs* Western blot	ELISA *vs* SDS-PAGE	ELISA *vs* native electrophoresis
Ficedula hypoleuca (1/8000)[c]	0·88***	0·01 NS[b]	–
Parus caeruleus (1/4000)[c]	0·85***	0·72***	0·08 NS
Parus caeruleus[a] (1/2000)[c]	0·93***	0·14 NS[b]	–
Lanius meridionalis (1/32 000)[c]	0·75***	0·95***	0·97***
Lanius collurio (1/16 000)[c]	0·91***	0·81***	0·81***
Athene noctua (1/16 000)[c]	0·88***	0·90***[b]	0·38*
Falco tinnunculus (1/8000)[c]	0·76***	0·54**[b]	0·05 NS
Falco tinnunculus[a] (1/8000)[c]	0·52**	0·45*	0·20 NS

[a]Nestlings.
[b]Correlation achieved using only data from immunoglobulin light chains.
[c]Optimum dilution of serum in ELISA.
*$P < 0·05$; **$P < 0·01$; ***$P < 0·001$; NS, not significant.

Once the linear range of the sigmoidal curve was achieved for each species, we chose the data obtained using the serum dilution nearest to the centre of the linear range. In order to control for the possible background of the technique, we removed the antichicken antibody step and confirmed that the absorbances achieved were always lower than 0·15. On the other hand, we coated some ELISA wells with ovoalbumin to control the binding of the polyclonal antichicken to this protein and absorbances were always lower than 0·2. However, these controls are not useful for detecting where the antibody binds. For this purpose, it is necessary to use the Western blot technique which allows observation of the bonding of the antibody.

Results

Twelve samples of each species were analysed by ELISA, Western blot, SDS-PAGE and native electrophoresis. Owing to the lack of sufficient serum in individuals from *Ficedula hypoleuca* and nestlings of *Parus caeruleus*, native electrophoresis could not be carried out. ELISA data were achieved using a certain optimum dilution of serum (Table 2). SDS-PAGE data were obtained by marking out the area corresponding to heavy and light immunoglobulin chains, but this procedure was very difficult to perform for some species because immunoglobulin chains and other proteins overlapped (i.e. Fig. 1a). Native electrophoresis data were obtained marking out the area corresponding to the γ-globulin fraction (i.e. Fig. 1b). Marking out this fraction is subjective and even impossible to do for some species. Validation of antichicken antibodies was made by Western blot. This antibody detected proteins corresponding to the apparent molecular weight of heavy and light immunoglobulin chains from different species (Fig. 2a) and also from chicken (Fig. 2b). As can be seen, the molecular weight of heavy chains is more variable among species than the molecular weight of light chains. On the other hand, ELISA results and data obtained exclusively from light chains by Western blot were also positively

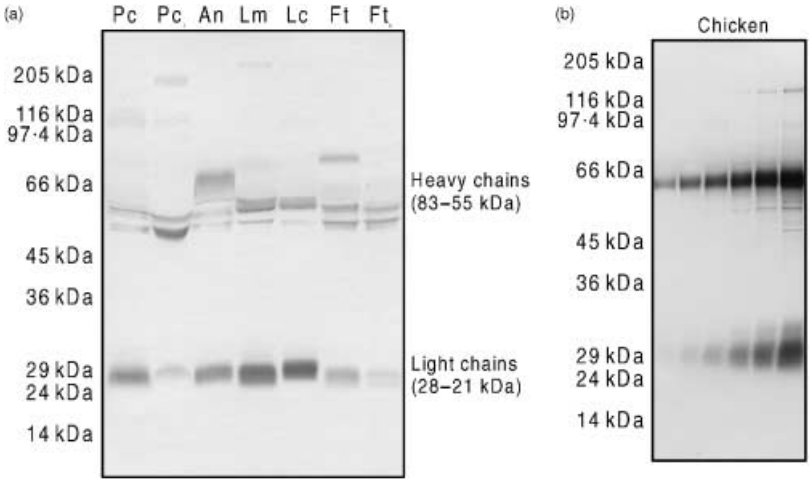

Fig. 2. (a) Study of specifity of antichicken antibodies by means of Western blot in all selected species (Left to right: molecular weights; Pc (left column) = adults of *Parus caeruleus*; Pc (right column) = nestlings of *Parus caeruleus*; An = *Athene noctua*; Lm = *Lanius meridionalis*; Lc = *Lanius collurio*; Ft (left column) = adults of *Falco tinnunculus*; Ft (right column) = nestlings of *Falco tinnunculus*). (b) Western blot using different dilutions of chicken serum (Left to right: molecular weights, serum dilutions 1/2560, 1280, 640, 320, 160, 80).

correlated. This result is a good control to determine that antichicken antibodies did not bind to albumin, which has a molecular weight similar to heavy chains.

Quantification of heavy and light immunoglobulin chains on blots was carried out as in SDS-PAGE or native electrophoresis (i.e. Fig. 1c). Once we had obtained results from the four methods, we performed correlation analyses (Table 2). ELISA results from all species were significantly correlated with Western blot data. However, data achieved by N-PAGE were only significantly correlated with ELISA when the γ-globulin fraction was easily marked out (i.e. genus *Lanius* and *Athene*). SDS-PAGE data were significantly correlated with results from ELISA in all species except *Ficedula hypoleuca* and nestlings of *Parus caeruleus*. In addition, ELISA data were correlated only with data obtained from light chains for some species (*Athene noctua* and adults of *Falco tinnunculus*).

Discussion

Detection of serum immunoglobulins from wild birds by means of ELISA using a non-specific antibody such as an antichicken antibody is possible, but some considerations should be made. Firstly, detection of total immunoglobulins needs a previous study to determine the sigmoidal curve and choice of a serum dilution included in the linear range (optimum dilution). This procedure should be made for all species because the affinity of the antichicken antibody could be different in each species. Later, the ELISA data must be validated by detecting immunoglobulins from the same sera by other methods. In this sense, Western blot is the best technique to validate the data obtained with ELISA because we can visualize the protein band recognized by antichicken antibodies. A positive correlation between ELISA and Western blot data confirms the validity of our results (see Table 2). To use electrophoretic methods to validate ELISA data is possible, but there are some problems. Native electrophoresis is a useful method to detect the γ-globulin fraction, but defining this fraction on densitometric profiles is very difficult in some species. For this reason, ELISA results were correlated with N-PAGE data only in three species. On the other hand, SDS-PAGE has a clear disadvantage when defining both heavy and light immunoglobulin chains, owing to overlap with other proteins of similar molecular weight. In this sense, only in species with low overlap did SDS-PAGE results correlate well with ELISA data (*Parus caeruleus*, *Lanius meridionalis*, *Lanius collurio* and nestlings of *Falco tinnunculus*). In some species it was impossible to separate the different immunoglobulin chains, and SDS-PAGE data did not correlate with ELISA results. On the other hand, in some species (*Athene noctua* and adults of *Falco tinnunculus*) the heavy chains were difficult to mark but the light chains were not. In these cases, although SDS-PAGE data (heavy and light chains) did not correlate well with ELISA results, SDS-PAGE results achieved exclusively from light chains (easier to mark out) correlated well with ELISA data (Table 2).

Secondly, studies performed to detect specific antibodies (antibodies produced against particular antigens) by ELISA require two control steps: (i) validate antichicken antibody specificity using Western blot (as described above) and (ii) check whether antichicken antibodies recognize the coated antigen on ELISA plates (non-specific reaction). Several studies have used indirect ELISA to detect specific antibodies produced against certain antigens, and have used different protocols to comply with step (ii). Thus, Svensson *et al*. (1998) used a rabbit antichicken conjugated antibody to measure antigen-specific antibodies. Fair *et al*. (1999) and Fair & Myers (2002) used a competitive ELISA where the plates were coated with Newcastle

disease virus (NDV) antigen. Serum from test birds was added, and then a conjugated monoclonal chicken antibody against NDV was added in excess to bind to the remaining NDV antigen. In Hasselquist *et al*. (1999) and Ilmonen *et al*. (2000, 2002), the set-up was an indirect ELISA based on coating with the antigen, adding the test plasma where antigen-specific antibodies bind to the antigen, then adding a rabbit anti-Redwinged Blackbird IgG antibody that binds to the passerine antibodies in the plasma, and finally adding an anti-rabbit IgG conjugated antibody to induce a colour reaction that estimates the amount of antigen-specific passerine antibodies that have bound to the antigen. Only in one case was the specificity of the secondary antibody validated by Western blot (Phalen *et al*. 1995). However, to our knowledge no other study has validated ELISA data with other immunological or electrophoretic techniques, thus fulfilling step (i).

To conclude, we have shown: (i) by means of ELISA, detection of serum immunoglobulins from wild birds using antichicken antibodies is possible because our results show a significant correlation with Western blot data in all selected species and (ii) electrophoretic methods to detect immunoglobulins have some problems in defining the immunoglobulin fraction in some species and they are more laborious to perform. We recommend that the specificity of antichicken antibodies is validated by Western blot and the optimum dilution of serum for other species is obtained before quantifying immunoglobulins.

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