Comparison of Techniques of Detecting Immunoglobulin-binding Protein Reactivity to Immunoglobulin Produced by Different Avian and Mammalian Species

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ABSTRACT

The rationale of this study was to use several immunological assays to investigate the reactivity of immunoglobulin binding protein (IBP) to immunoglobulins from various avian and mammalian species. The IBP studied were Staphylococcal protein A (SpA), Streptococcal protein G (SpG), Peptostreptococcal protein L (SpL) and recombinant protein LA (SpLA). The various immunological techniques used were double immunodiffusion (Ouchterlony technique) that tested positive high protein reactivities, direct and competitive enzyme-linked immunosorbent assays (ELISAs) that tested moderate and low positive protein binding capacities, respectively. In addition to sandwich ELISAs, immunoblot analyses and Ig-purification by SpA-affinity chromatography, which were sensitive tests and helpful in the screening and confirmatory tests were also used. The Ouchterlony technique showed that compared to the other proteins, SpLA had the highest range of reactivity with animal sera and purified immunoglobulins while SpL was least reactive. With the direct ELISA, SpL reacted with the raccoon sera, rabbit IgG and with IgY from bantam hens and pigeons. While with the direct ELISA, SpA reacted with sera from skunk, coyote, raccoon, mule, donkey and human. The sandwich ELISA revealed high reactivity of both SpG and SpLA with mammalian sera titres ranging from 1:32 (raccoon serum) to 1:1024 (mule and donkey sera). These results suggest that IBP can be used for the detection of immunoglobulin using various immunological assays and this is important for the diagnosis of infectious diseases in animal and bird populations studied and in the purification of immunoglobulins.

Keywords: Immunoglobulin binding protein (IBP), staphylococcal protein A (SpA), streptococcal protein G (SpG), peptostreptococcal protein L (SpL), recombinant protein LA (SpLA)

Comparación de las Técnicas de Detección de la Reactividad de la Proteína De Unión de la Inmunoglobulina Frente a la Inmunoglobulina Producida por Diferentes Especies Aviarias y Mamíferas

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RESUMEN

El fundamento de este estudio radica en el uso de varios ensayos inmunológicos para investigar la reactividad de la proteína de unión de la inmunoglobulina (IBP) frente a las inmunoglobulinas de varias especies aviarias y mamíferas. Las proteínas IBP estudiadas fueron la proteína estafilocócica A (SpA), la proteína estreptocócica G (SpG), la proteína peptoestreptocócica L (SpL), y la proteína recombinante LA (SpLA). Las varias técnicas inmunológicas usadas fueron: la inmunodifusión doble (técnica de Ouchterlony) para examinar las reactividades positivas de la proteína alta; el ensayo por inmunoabsorción ligado a enzimas (ELISA), de tipo directo y competitivo, para examinar la capacidad de realizar uniones positivas de proteína moderada y baja, respectivamente, además del ensayo ELISA 'Sándwich', los análisis inmunoblot, y la purificación de IgG, mediante cromatografía de afinidad, los cuales fueron pruebas sensibles y útiles en el tamizaje y las pruebas de confirmación. La técnica de Ouchterlony mostró que – en comparación con otras proteínas – la SpLA tenía el grado más alto de reactividad con los sue-

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ros animales y las inmunoglobulinas purificadas, mientras que la SpL fue la menos reactiva. Con el ELISA directo, la SpL reaccionó con los sueros de mapache, la IgG de conejo, así como con la IgY de palomas y gallinas de Bantam, en tanto con el ELISA directo, la SpA reaccionó con sueros de mofeta, coyote, mapache, mula, asno y seres humanos. ELISA "sándwich" reveló una alta reactividad tanto de SpG como de SpLA, con títulos séricos mamíferos que iban desde 1:32 (suero de mapache) hasta 1:1024 (sueros de mula y de asno). Estos resultados sugieren que la proteína de unión IBP puede usarse en la detección de la inmunoglobulina usando varios ensayos inmunológicos, lo cual es importante para el diagnóstico de enfermedades infecciosas en las poblaciones animales y aviarias bajo estudio, así como para la purificación de inmunoglobulinas.

Palabras claves: Proteína de unión de la inmunoglobulina (IBP), proteína estafilocócica A (SpA), proteína estreptocócica G (SpG), proteína peptoestreptocócica L (SpL), proteína recombinante LA (SpLA).

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INTRODUCTION

The binding of immunoglobulin-binding protein (IBP) such as staphylococcal protein A (SpA), streptococcal protein G (SpG), peptostreptococcal protein L (SpL) and recombinant protein LA (SpLA) to the sera and immunoglobulins (Igs) of animal species is well known for many mammalian species (1–4). However, the reactivity of immunoglobulin-binding protein for a number of mammalian and avian Ig molecules has not been previously reported. To investigate the reactivity of other immunoglobulin molecules from several other sources is very critical and is important since these bacterial proteins can be used as immunological tools in immunoassays including enzyme-linked immunosorbent assays (ELISA) and western blotting for the immunodiagnosis of infectious diseases and also purification of IgG molecules and their fragments (5–7). The reactivity of bacterial Ig receptors for immunoglobulins from a variety of mammalian and avian species was assessed by a number of immunological procedures including doubled immunodiffusion, ELISA and others assays.

Double immunodiffusion in Agar (Ouchterlony) technique was first described by Ouchterlony in 1953 (8). It is based on the principle that antigen and antibody diffuse through a semisolid medium (usually agar) and form stable immune complexes at optimal concentrations, which then can be visualized and analysed. The formation of a single precipitation line between an antigen and its corresponding antiserum can be utilized as a rough measure of the antigen concentration. Double immunodiffusion is frequently used for immunodiagnosis and determination of the titre of specific antibodies against several antigens (9).

Enzymatic immunoassays (EIA) belong to the third generation of immunological methods. They have had diverse applications in biomedical investigations, immunodiagnosis, immunogenicity and other studies. Enzyme-linked immunoabsorbent assay, first described by Engvall *et al* (10), provides a safe and simple method of measuring antigen-specific and total Ig concentration. Enzyme-linked immunoabsorbent assay falls under the category of heterogeneous immunoabsorbent assays in which the antigen to be detected is either directly or indirectly physically attached to a solid phase. Different categories of ELISA have been described (11) based on the entity to be detected or quality to be determined antigen or antibody (qualitative, quantitative or semi-quantitative), the set-up of the assay (direct, indirect, competitive, inhibitory, non-competitive, two-site sandwich, or amplified assays), the nature of the substrate employed (fluorogenic, chromogenic, luminogenic) and the amount of reactants used (macro-ELISA, performed in a tube, micro-ELISA employing a microtitre plate, or micro-ELISA needing micro quantities (5 µl) of sample performed in Terasaki trays – the Terasaki ELISA). The usefulness of an ELISA depends upon the coating efficiency and the reproducibility of the coat. The use of a high ionic strength buffer (0.1 M Na_2CO_3, pH 9.6) for efficient coating of antibodies has been previously suggested (10).

The aim of using several immunological procedures, *eg* double immunodiffusion and ELISAs, in this research was to compare and assess the degree of binding capacity between immunoglobulin-binding protein and different sources of Ig-containing samples. These findings may strongly confirm that IBP can be used for the detection of immunoglobulin using various immunological assays and this is important for the diagnosis of infectious diseases in animal and bird populations studied and in the purification of immunoglobulins.

MATERIALS AND METHODS

Immunoglobulin Y isolation and determination by direct enzyme-linked immunosorbent assay

The IgY fraction was isolated from the egg yolks of a variety of birds including chicken, bantam hen, guinea hen, quail, goose, duck, pigeon, parakeet, cattle egret, pheasant and ostrich. The IgY fraction was isolated by the chloroform-polyethylene glycol (PEG) method (12). The eggs were washed with warm water and the egg yolk was separated from the egg white. The membrane was broken and the egg yolk collected and diluted 1:3 in phosphate buffered saline (PBS), pH 7.4. To one-third of the egg yolk mixture, an equal volume of chloroform was added; the mixture was then shaken and centrifuged for 30 minutes, 1000 x g, room temperature (RT). The supernatant was decanted and mixed with PEG 6000 (12%, w/v), stirred and incubated for 30 minutes at RT. The mixture was

then centrifuged as described above. The precipitate containing IgY was dissolved in PBS (pH 7.4) at a volume equivalent to one-sixth of the original volume of the egg yolk and dialysed against 1 L of PBS (pH: 7.4 for 24 hours at 4 ˚C). The IgY was removed from the dialysis tubing. Immunoglobulin Y concentration was determined by the Bradford method (13). Immunoglobulin Y samples were stored at –20 ˚C.

A direct ELISA was used to determine the presence of avian egg yolk IgYs as follows: 96 well microtitre plates were coated overnight at 4 ˚C with 100 µg of duplicates of each IgY sample in carbonate-bicarbonate buffer pH 9.6. Plates were washed four times (4x) with 150 µl PBS-Tween 20 buffer (Sigma-Aldrich Co, St Louis, Missouri). Then 50 µl of a commercial anti-chicken IgY-horseradish peroxidise (HRP), Sigma-Aldrich diluted 1:30 000 (according to manufacturer's instructions) in PBS-nonfat milk, was added to each well and incubated for one hour at RT. The plates were washed 4x with PBS-Tween. Fifty µl of 3 mg/ml o-phenylenediamine solution (OPD) was added and the plates were incubated 15−20 minutes at RT. The reaction was stopped with 25 µl of 3M sulphuric acid (H_2SO_4) solution. The plates were read in a microplate reader at 492 nm.

Binding properties of bacterial immunoglobulin receptors by double immunodiffusion (Ouchterlony) technique

The binding of SpLA, SpL and SpG with animal sera, avian IgY, avian egg whites and purified IgG were investigated by double immunodiffusion as previously described (8). Briefly, 1% agarose gels were prepared and wells cut into the gel using a template. Initially, aliquots of 25 µl each of SpLA, SpL or SpG at 1 µg/µl were applied to the centre well. The peripheral wells were filled with 25 µl each of IgY (30 µg/µl), avian egg white diluted 1:2 in PBS pH 7.4, or animal serum. The gels were incubated at RT for 48−72 hours and then examined for precipitin lines. Human serum and human IgG were included as positive controls. The positive results were taken as the presence of precipitin line/s and negative results, the absence of precipitin lines. The experiments were repeated using concentrations of each bacterial Ig receptor and animal serum or purified immunoglobulin ranging from 1−51 µg/µl.

Direct enzyme-linked immunosorbent assay for investigating SpL binding to purified mammalian and avian immunoglobulins

A direct ELISA was used to study the interaction of SpL with different concentrations of purified immunoglobulins. The 96 well microtitre plates were coated overnight at 4 ˚C with 50 µl of serial doubling dilutions of purified rabbit and mouse IgG in carbonate-bicarbonate buffer pH 9.6 (Sigma-Aldrich Co, St Louis, Missouri). Plates were washed 4x with 150 µl PBS-Tween 20 buffer (Sigma-Aldrich Co, St Louis, Missouri). Then, 100 µl of SpL-HRP conjugate diluted 1:1000 in PBS-non-fat milk was added to each well and incubated for one hour at RT. The plates were washed 4x with PBS-Tween; 100 µl of 3 mg/ml o-phenylenediamine solution (OPD) was added and the plates were incubated 15−20 minutes at RT. The reaction was stopped with 50 µl of 3M H_2SO_4 solution. The plates were visually assessed for the development of colour (indicating a positive result) and read in a microplate reader at 492 nm. The assay was repeated three times and the inter-assay coefficient of variations (CV %) calculated. A positive result was taken as equal or above the cut-off point (0.09) calculated as follows:

Cut-off point = 2 x (OD of negative controls); where OD is the optical density.

Direct enzyme-linked immunoabsorbent assay for investigating SpL binding to animal and bird sera, avian IgY, avian egg whites and purified cat IgG

The SpL interactions with animal and bird sera, avian IgY, avian egg whites and purified cat IgG were studied by direct ELISA in a similar procedure to that already described above. The microplates were coated overnight at 4 ˚C with 20 µl serum from skunk, coyote or raccoon serum; 50 µl duck pooled sera, 25 µl human sera, 25 µg cat IgG (Sigma-Aldrich Co, St Louis, Missouri), 62.5 µg chicken IgY (Sigma-Aldrich Co, St Louis Missouri); 100 µl of egg whites, 1 µg of purified human IgG, 50 µg of bantam hen IgY or domestic hen IgY. Samples were diluted in 50 µl of coating buffer pH 9.6 (Sigma-Aldrich Co, St Louis, Missouri).

Competitive enzyme-linked immunosorbent assay for investigating SpL binding to mammalian and avian immunoglobulins

This ELISA was based on the theory that antibodies present in different samples would compete with human IgG for binding to SpL, resulting in inhibition of human IgG-SpL interactions. The samples tested were commercially prepared pooled sera from skunk, coyote, raccoon, duck, and also commercially prepared purified immunoglobulins from cat and chicken (Sigma-Aldrich Co, St Louis, Missouri). Microplates were coated with 50 µl of commercial human IgG (1 µg/well overnight at 4 ˚C). Serial doubling dilutions (1:4 to 1:1024) of 30 µl of each sample were made in a separate microplate to which 30 µl of the conjugate SpL-HRP diluted 1:1000 in non-fat milk was added. The microplates were incubated for one hour at RT and then 50 µl of each sample was transferred to the human IgG coated microplate and incubated for one hour. The microplates were then washed four times with PBS-Tween 20 buffer (Sigma-Aldrich Co, St Louis, Missouri), and 50 µl of the substrate OPD (3 mg/ml) was added to each well and incubated at RT for 15 minutes. The reaction was stopped with 3M H_2SO_4 and the microplates were visually assessed and read at 492 nm. The percentage of the binding inhibition (I%) of the SpL-human IgG interactions by different samples was calculated using the formula:

$$I\% = 100 - \left\{ \frac{(\text{Mean OD of sample}) - (\text{Mean OD of blank})}{(\text{Mean OD 100\% SpL binding to human IgG}) - (\text{Mean OD of blank})} \right\} \times 100$$

Investigation of SpA binding to serum, egg white and purified immunoglobulin of various animals in direct enzyme-linked immunosorbent assay
The direct ELISA described above for studying SpL interactions was also used to measure the SpA binding to animal sera, IgY, egg whites and purified immunoglobulins using a SpA-HRP conjugate at 1:1000 (Sigma-Aldrich Co, St Louis, Missouri). The cut-off point was 0.09.

Competitive enzyme-linked immunosorbent assay for investigating SpA binding to mammalian and avian immunoglobulins
The competitive ELISA described above for studying SpL interactions was used to measure the SpA binding to mammalian and avian immunoglobulins using a SpA-HRP conjugate (Sigma-Aldrich Co, St Louis, Missouri).

Direct enzyme-linked immunosorbent assay for investigating SpLA binding to mammalian and avian immunoglobulins
The SpLA-direct ELISA was carried out as described above for the protein L with modifications. A SpLA-HRP conjugate (Sigma-Aldrich Co, St Louis, Missouri) diluted 1:1000 was used. The cut-off point was 0.09.

Purification of immunoglobulins from animal sera and avian eggs
A commercially prepared protein-A antibody purification kit (Sigma-Aldrich Co, St Louis, Missouri) based on affinity chromatography was used to purify IgG from the sera of skunk, coyote, raccoon, mule, horse, donkey, dog plasma, IgY from ostrich, bantam hen and duck egg yolks and ostrich IgM from the ostrich egg white. The procedure was performed according to the manufacturer's instruction.

Protein G-Protein LA sandwich enzyme-linked immunosorbent assay for investigating the SpG interactions with mammalian and avian purified Ig molecules and sera
The capability of SpG to bind to IgGs of certain species was determined by sandwich ELISA. Fifty µl of serial dilutions of human sera and purified immunoglobulins were added to microplates coated with 1 µg/well of SpG. After incubation at RT for one hour, microplates were washed (4x with PBS-Tween 20 buffer, pH 7.4). Fifty µl of SpLA-HRP diluted 1:1000 in PBS non-fat milk pH 7.4 was added and the microplates incubated for one hour at RT. Microplates were washed as above, 50 µl OPD added and the microplates kept in the dark at RT for 15 minutes. The reaction was stopped with 50 µl of 3M H_2SO_4 solution and the microplates were examined visually for colour development and read at 492 nm. Triplicates of serial doubling dilutions from 1:8 to 1:1024 of sera from skunk, coyote, raccoon, mule, donkey, duck, turtle (control); commercially prepared IgG (controls): cat IgG, human IgG, mouse IgG, goat IgG, chicken IgY and rabbit IgG were also tested in this experiment which was repeated three times.

Immunoblot analyses for investigating SpLA binding to purified mammalian and avian immunoglobulins
Aliquots of 3−5 µg/µl purified Ig were applied to the gel as described above. Gels were transferred to nitrocellulose membranes (Immobilon-Nc, pore size 0.45 µm, Sigma-Aldrich Co, St Louis, Missouri) during 75 minutes at 40 mAmps using a semi-dry electroblotter, HEP-1 Model, Owl Scientific Inc. The running buffer contained 25 mM Tris, 192 mM glycine pH 8.3 and 20% methanol. The nitrocellulose membranes were blocked overnight in 10% non-fat skim milk in PBS with 0.05% Tween-20 pH 7.4 and then washed 4x, 10 minutes with PBS-Tween 20. Recombinant protein LA (Sigma-Aldrich Co, St Louis, Missouri) at a concentration of 5 µg/ml was added to membranes. After incubation at 4 ˚C overnight, the nitrocellulose membranes were washed as above. A secondary antibody (rabbit anti-chicken IgY horseradish peroxidase, Sigma-Aldrich) was added at a 1:15 000 dilution. This was incubated for one hour at room temperature and washed as above. Tetramethyl-benzidine (TMB) solution was added to the nitrocellulose membranes, which were then incubated in the dark for a few minutes, shaken gently and rinsed thoroughly in de-ionized water to stop the blotting process and dried. Alternatively, Ig samples were transferred to nitrocellulose membranes and directly probed using SpLA-HRP (diluted 1:5000) and TMB. This system was mainly used for detecting avian Ig.

RESULTS

The affinity of immunoglobulin-binding protein for Ig molecules, sera and avian egg components as well as immunoglobulins from different mammals using several test techniques and to different bacterial proteins and immunoglobulins are compared and summarized in Tables 1 to 5. In general, the degree of reactivity was lower among Ig-samples of birds compared to that of the mammals. It suggests that the affinity shown in these interactions was directly related to the high degree of development, organization and versatility of the immune system observed in mammalian species. Human, mouse, pig, rabbit, skunk and raccoon showed a total affinity score higher than seven (based on the sum of the individual protein interactions). Avian immunoglobulins generally showed scores lower than four. A novel finding was the high affinity score observed for the ostrich IgY than the other avian species used in this study. Direct ELISA detected the IgY of several avian species as illustrated in the Figure. It proved that the chloroform-PEG technique was successful for isolating IgY from the egg yolk of several birds including guinea hen, pheasant, duck, bantam hen, chicken, ostrich, pigeon, cattle egret, parakeet, goose and quail.

Comparison of the reactivity of peptostreptococcal protein (SpL) using the various immunological techniques are shown in Table 1. The reactivity of SpL proteins originating

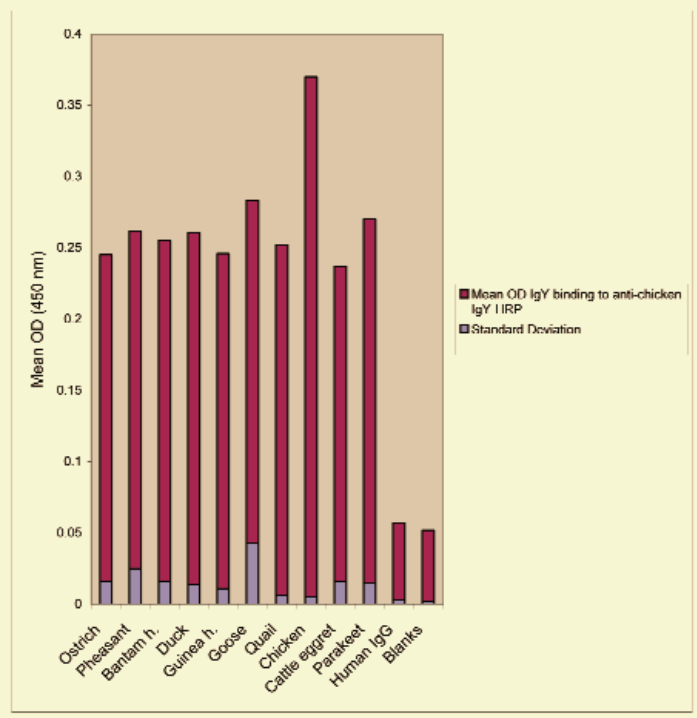

Figure: Direct enzyme-linked immunosorbent assay for the detection of avian IgYs extracted from the egg yolk of several birds by the Polson method. Duplicate samples of avian IgY were detected. The cut-off point is 0.1.

from different mammals and birds using different techniques revealed that Ouchterlony technique performed very poorly in comparison to the other techniques. Oucherterlony only detected reactivity of SpL in samples of human serum, human IgG and pig IgG. Competitive ELISA techniques detected the most reactivity of the SpL proteins in the samples assessed from IgG from cat and humans, sera from humans, skunk, coyote and raccoon. Direct ELISA technique faired much better in detecting SpL protein reactivity to immunoglobulin originating from the birds than the other sources.

A novel finding was the reactivity of SpL with ostrich egg white. Chicken IgY and turtle serum did not react with SpL and they were used as a negative control. The inhibition of the binding of SpL to human IgG by sera or immunoglobulins from a variety of animal sources in the competitive ELISA showed the SpL binding with sera and immunoglobulins of some mammalian and avian species had different reciprocal titres and percentage of binding inhibition. Immunoglobulins from human and raccoon sera had reciprocal titre of 512, but their percentages of binding inhibition were 14.8 and 24.7%, respectively. There was no reaction with the turtle sera. The competitive ELISA reciprocal titres of purified Ig molecules and other Ig sources for cat IgG, chicken IgY and pigeon egg whites were 32, 8 and 8, but their binding inhibition were 30.8%, 34.6% and 23.5%, respectively.

The binding of peptostreptococcal protein L (SpL) to mammalian sera by direct ELISA revealed positive reactions (which was determined by colour development and measured by their optical densities) only for human and raccoon sera. The colour that was developed for the sera had optical density of 0.21 and 0.11 with standard deviation values of 0.01 and 0.001, respectively. The cut-off point for all the optical density values was 0.09; any value below 0.09 was negative but any value equal and above 0.09 was positive. The binding of pep-

Table 1: Comparison of the reactivity of pepstreptococcal protein (SpL) using various immunological techniques

Samples	Ouchterlony[A]	Direct ELISA[B]	Competitive ELISA[C]	Reactivity score
Cat IgG	–	–	+	1
Skunk serum	–	–	+	1
Coyote serum	–	–	+	1
Raccoon serum	–	+	+	2
Human serum	+	+	+	3
Human IgG	+	+	+	3
Pig IgG	+	Na	Na	3
Goat IgG	–	–	Na	0
Sheep IgG	–	–	Na	0
Turtle serum	–	–	–	0
Chicken IgY	–	–	+	1
Ostrich IgY	–	+	Na	2
Bantam IgY	–	+	Na	2
Duck IgY	–	–	Na	0
Pigeon IgY	–	+	Na	2
Ostrich egg white	–	+	Na	2
Duck egg white	–	–	Na	0
Pigeon egg white	–	–	+	1
Duck serum	–	–	–	0

A = Determines the presence of precipitin line; B = Cut-off point < 0.09; C = Capacity of Igs to inhibit the SpL binding to human IgG in dilutions of 1:4 or higher; – = Negative; + = Positive; Na = Not assessed; 0 = No binding affinity shown in most sensitive test performed; 1 = low affinity; 2 = Moderate affinity; 3 = High affinity

tostreptococcal protein L to avian IgY by direct ELISA revealed colour development for only bantam and pigeon immunoglobulin Y. These two specimens had optical density values of 0.11 and 0.16 with standard deviations of 0.007 and 0.008, respectively with a cut-off point of 0.09. The binding of peptostreptococcal protein L to avian egg white sample by direct ELISA had no reaction for bantam and pheasant. But there were colour development for ostrich, duck and pigeon. Their OD values were 0.11, 0.09 and 0.09, respectively.

Table 2 shows the results for investigation of staphylococcal protein A (SpA) reactivity with serum, egg white and immunoglobulin of various animals in direct ELISA revealed positive reaction (manifested by colour development) for the sera from skunk, coyote, raccoon, mule, donkey and humans. There were no binding reactions observed for the sera from turtle. The OD observed for the sera from the turtle was 0.05, (STD 0.0001); but from the other animals the OD observed were in the region of 0.12 (donkey) to 0.26 (human). The investigation of binding of staphylococcal protein A to avian IgY by direct ELISA revealed positive reaction for ostrich, bantam, duck and pigeon. There were no reactions for chicken and quail. The optical density values for both the ostrich and bantam were 0.09, while for the duck and pigeon, they were 0.13 and 0.18, respectively. The binding of staphylococcal protein A to avian egg whites by direct ELISA had positive reactions for only the ostrich and duck with an OD value of 0.11 and 0.09, respectively. But there were no reactions for the pigeon, bantam and chicken.

The results of subsequent binding experiments with sera from other animal species, avian IgY and avian egg whites revealed that in the direct ELISA, there was no binding with the recombinant protein LA (SpLA) immunoglobulin from chicken and turtle (Table 3). All the other animal species, avian immunoglobulin Y and avian egg white reacted posi-

Table 2: Comparison of the reactivity of Staphylococcus aureus protein A (SpA) using various immunological techniques

Samples	DID[A]	Competitive ELISA[B]	PIC[C]	Reactivity score
Cat IgG	+	Na	Na	3
Skunk serum	+	+	+	3
Coyote serum	+	+	+	2
Raccoon serum	+	+	+	3
Human serum	+	+	+	3
Donkey serum	+	Na	+	2
Mule serum	+	Na	+	3
Dog plasma	Na	Na	+	3
Horse serum	Na	Na	+	3
Goat IgG	+	Na	Na	2
Sheep IgG	+	Na	Na	2
Human IgG	+	+	+	3
Turtle serum	–	–	–	0
Chicken IgY	–	–	–	0
Ostrich IgY	+	Na	+	2
Bantam IgY	+	Na	+	1
Duck IgY	+	Na	Na	2
Pigeon IgY	+	Na	Na	2
Ostrich egg white	+	+	+	2

A = Double immunodiffusion/Direct ELISA, cut-off point =0.09; B = Capacity of Igs to inhibit the SpA binding to rabbit IgG in dilutions of 1:4 or higher; C = PIC, purification of immunoglobulins by affinity chromatography; - = Negative; + = Positive; Na = Not assessed; 0 = No binding affinity shown in most sensitive test performed; 1 = low affinity; 2 = Moderate affinity; 3 = High affinity

Table 3: Comparison of the reactivity of recombinant protein (SpLA) using various immunological techniques

Samples	Ouchterlony[A]	Direct ELISA[B]	Immunoblot analysis[C]	Reactivity score
Cat IgG	+	+	+	3
Skunk serum	+	+	+	3
Coyote serum	–	+	+	2
Raccoon serum	+	+	+	3
Human serum	+	+	+	3
Human IgG	+	+	Na	3
Donkey serum	+	+	+	3
Mule serum	+	+	+	3
Turtle serum	–	–	–	0
Chicken IgY	–	–	–	0
Ostrich IgY	–	+	+	2
Bantam IgY	–	+	+	2
Duck IgY	–	+	+	2
Pigeon IgY	–	+	Na	2
Ostrich egg white	–	+	+	2
Duck egg white	–	+	Na	2
Pigeon egg white	–	+	Na	2

A = Determines the presence of precipitin line; B = Cut-off point = 0.09; C = Immunoblot analysis, purified Igs from different sera and avian eggs were probed with SpLA – HRP; – = Negative; + = Positive; Na = Not assessed; 0 = No binding affinity shown in most sensitive test performed; 1 = low affinity; 2 = Moderate affinity; 3 = High affinity

tively suggesting that there was binding to the SpLA. Generally, all the immunological techniques had very high binding affinity or reactivity with SpLA. The range of colour development (determined by the OD values) were as follows: bantam and duck 0.09, ostrich and mule 0.11, donkey 0.12, pigeon 0.14, coyote 0.20, skunk 0.24, and raccoon 0.25. Sera from human had the OD value or colour reaction of 0.27 indicating that it had the highest binding. The OD values for the chicken and turtle were 0.06 and 0.05, respectively.

In Table 4, streptococcal protein G (SpG) binding to purified immunoglobulin molecules and sera by protein G and protein LA sandwich ELISA in several dilutions of SpG and SpLA-HRP showed high reactivities for human, rabbits and mouse IgG. Good reactivity was observed with the mammalian sera with titres ranging from 1:32 (raccoon serum) to 1:1024 (mule and donkey sera). In contrast, the avian Igs from duck serum and pigeon egg white reacted poorly in this assay. A comparison of reactivity of SpG using sandwich ELISA and Ouchterlony revealed high binding affinity for rabbit, pig and human IgG, sera from skunk, human and donkey.

Table 4: Comparison of the reactivity of streptococcal protein G (SpG) using various immunological techniques

Samples	Ouchterlony[A]	Sandwich ELISA[B]	Reactivity score
Cat IgG	–	+	1
Rabbit IgG	+	+	3
Goat IgG	–	+	2
Pig IgG	+	Na	3
Sheep IgG	+	Na	3
Skunk serum	+	+	3
Coyote serum	–	+	1
Raccoon serum	–	+	1
Human serum	+	Na	3
Human IgG	+	+	3
Donkey serum	+	+	3
Turtle serum	–	Na	0
Chicken IgY	–	–	0
Ostrich IgY	–	Na	2
Bantam IgY	–	Na	0
Pigeon egg white	–	Na	0
Duck serum	–	–	0
Mouse IgG	+	+	3

A = Determines the presence of precipitin line; Sandwich ELISA = 1ug/well of SpG reacts with 25 ug of purified IgG or with dilutions 1:8 of several samples; – = Negative; + = Positive; Na = Not assessed; 0 = No binding affinity shown in most sensitive test performed; 1 = low affinity; 2 = Moderate affinity; 3 = High affinity

A proper assessment of the affinity of immunoglobulin-binding protein for immunoglobulins was possible by the use of different immunological techniques with different degrees of sensitivity (Table 5). For example, the double immunodiffusion was applicable at higher levels of binding affinity. Direct and sandwich ELISA were useful for moderate interactions. Competitive ELISAs were the most sensitive test for the determination of the low level of protein reactivity. The purification of several Ig molecules from Ig-containing samples by SpA-affinity chromatography was a confirmatory test of the SpA and SpLA reactivity to several Igs.

Table 5: Comparison of the reactivity of bacterial Ig receptors with some mammalian and avian immunoglobulins

Samples	SpG[A]	SpL[B]	SpA[C]	Reactivity score
Pig IgG	3	3	3	9
Rabbit IgG	3	2	3	8
Goat IgG	3	0	2	5
Sheep IgG	3	0	2	5
Human IgG	3	3	3	9
Mouse IgG	3	3	3	9
Cat IgG	1	1	3	5
Skunk sera	3	1	3	7
Coyote sera	2	1	3	6
Raccoon sera	1	3	3	7
Mule serum	3	1	2	6
Donkey serum	3	1	2	6
Ostrich IgY	2	1	2	5
Duck IgY	1	1	2	4
Duck serum	0	1	1	2
Bantam hen IgY	0	2	1	3
Chicken IgY	0	0	0	0
Turtle serum	0	0	0	0

SpG = Streptococcal protein G; SpL = Peptostreptoccocal protein L; SpA = Staphylococcal protein A; 0 = No binding affinity shown in most sensitive test performed; 1 = low affinity; 2 = Moderate affinity; 3 = High affinity; Total reactivity score is designated as follows:0 = no affinity, 1 – 3 = low affinity, 4 - 6 = moderate affinity and 7 – 9 = high affinity

DISCUSSION

The Polson method was efficient for the purification of IgY molecules from egg yolk of several avian species. These results indicate that there are closely antigenic relationships among avian egg yolk IgY molecules. It was shown by the capacity of an anti-chicken IgY-HRP conjugate to cross-react with the entire panel of IgYs. It confirmed previous reports of the cross-reactivity of anti-turkey IgY antibodies with IgY of chickens, ducks and geese (13, 14). In the present study the *Staphylococcus aureus* Protein A (SpA) proved efficient in the affinity chromatography purification of IgY isolated by the Polson technique (12), which did not yield IgY of sufficient purity. Similarly protein A-affinity chromatography was used by Higgins *et al* (15) to purify duck serum IgY.

The higher reactivity of SpLA shown in the Ouchterlony technique may be explained by its chimeric molecular structure (4). The negative results obtained with avian egg whites and IgY extracted from the egg yolk of different birds could be due to lack of sensitivity of the Ouchterlony technique or the failure of the three immunoglobulin-binding protein to bind to avian Ig molecules. Fischer and Hlinak in 2000 reported that SpA and SpG were not able to bind to the egg yolk IgY of turkey, moskovy duck and goose (16). However, the direct ELISA with its high sensitivity compared to the Ouchterlony showed that SpL, in fact, is capable of binding some avian IgY. De Chateau *et al* (3) reported that SpL binds to chicken IgY, however, the results of both the present and

previous studies did not confirm this report. The SpL binding to bantam hen IgY and the lack of SpL binding to chicken IgY reflect the diversity in Ig molecules between these closely related avian species. Reactivity of SpL with IgM present in the ostrich egg white was shown in the direct ELISA. This has not been previously documented in the literature. This novel finding could be important in the assessment of the primary immune response in the ostrich, which is an important source of meat and eggs for human consumption in some countries.

A novel finding was the high affinity score observed for the ostrich IgY than the other avian species used in this study. This suggests the existence of phylogenetic differences among avian species. Probably the ostrich humoral immune system possesses a higher level of development among these birds. To test this hypothesis future work should look at the sequence homology between ostrich and mammalian Ig molecules, Ig-receptors expressed on B cells, genes involved in the re-arrangement of immunoglobulins, reactivity and specificity to several antigens in this species.

Another novel finding pertaining to SpL binding in the direct ELISA was its non-reactivity with turtle serum. The direct ELISA with SpL also confirmed the failure of this bacterial protein to bind to goat and sheep IgG as was previously reported (3). The direct ELISA revealed novel interactions of SpA with Ig molecules present in the sera of raccoon, skunk, coyote and mule. These interactions are important since they add to the list of animal species in which SpA can be used in the detection and quantification of immunoglobulins and also in immunodiagnosis of infectious diseases. SpA binding to donkey and human serum has been previously reported (7). The reactivity of SpA with some avian immunoglobulins such as the bantam hen IgY has not been previously reported.

The purification of these immunoglobulins by affinity chromatography confirmed their ability to bind to SpA. The SpA affinity chromatography technology may be considered a feasible methodology for the production of highly purified IgG for therapeutic purposes in human and several animal species. For example, it has been previously reported in the literature of the successful application of intravenous highly purified human IgG preparations in the treatment of human and animal diseases (17–25).

Protein A is known to have a high binding capacity for rabbit IgG (5, 7). This property was applied in the SpA competitive ELISA, in which the degree of inhibition by Ig or serum correlates directly with their binding affinity for SpA. This assay confirmed the previous interactions, which had been observed in the direct ELISA. The SpA binding to raccoon serum was even stronger (at higher dilutions) than the well reported strong binding of SpA to human serum (26). This suggests that in raccoon serum, various subclasses of Ig molecules with high binding affinity for SpA may be present. Horseradish peroxidase labelled protein-A has been used for the detection of anti-rabies antibodies in several animal species (27).

The direct ELISA for SpLA reactivities with sera, avian IgY and egg whites showed the superiority of SpLA as an immunological tool. The binding capacity of SpLA surpassed that of SpA and SpL and it might prove more useful in the antibody detection assays. The results of this study also contributed to the list of large animals whose sera are known to react with protein LA. The reactivity of SpLA with mule and donkey sera reported here has not been previously documented. The fact that these large animals are widely used in the production of antisera accentuates the importance of this new finding.

The successful results of the immunoblots using SpLA strongly support the potential use of these immunoglobulin-binding proteins in a variety of immunoassays. The use of SpLA in the immunoblot analysis in this study allowed for the molecular weight (MW) characterization of immunoglobulins from a number of mammalian and avian sources. For example, the characterization of the MW of the heavy and light chains of ostrich IgY was in keeping with previous reports (28).

The results suggest that SpLA could be used in sero-epizootic studies in wild animals, such as raccoons and skunks. These animals provide important health threats in some countries in their capacities as reservoirs of zoonotic infection including rabies (29−31).

An innovative method, a SpG-SpLA sandwich ELISA, was very important in confirming the binding of SpLA to several different Ig molecules and sera. In addition, it revealed novel binding properties of streptococcal protein G, an important immunological tool, which might be used for detection of immunoglobulins in several species, including skunk and mule, for which there was no previous information. This is in fact the first report on the simultaneous interactions of immunoglobulins with three immunoglobulin-binding proteins: Proteins L, A and G *in vitro*. This showed that immunoglobulins could serve as bridges between protein G and protein LA.

CONCLUSION

These results suggest that IBP can be used for the detection of immunoglobulin using various immunological assays and this is important for the diagnosis of infectious diseases in the population of animals and birds studied and in the purification of immunoglobulins.

Author's Contributions:

AAJV and NMA designed the study. AAJV collected and analysed the data. AAJV, NMA and MPS interpretated the data. AAJV and PEA drafted the manuscript. All authors read and approved the final manuscript. All the authors declare no conflict of interest

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