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Abstract

Eight twenty-one week old White Leghorn Laying hens were used to produce polyclonal antibodies against antigens of four canine pathogens: *Pythium insidiosum*, *Lagenidium sp.*, *Basidiobolus ranarum,* and *Conidiobolus coronatus.* Two birds were allocated to each organism. Eggs were collected for 45 days and antibodies contained in the yolks of the eggs were extracted by dilution in a weak acid solution followed by ammonium sulfate precipitation. The presence of IgY in resultant precipitates was confirmed by SDS-PAGE and western immunoblot analysis. In addition, western immunoblot analysis indicated that the antibodies produced in each pair of hens were specific for the antigens of the organisms for which they were immunized. These antibodies will be used to develop immunohistochemical assays to aid in the diagnosis of diseases caused by the aforementioned organisms.

Background

Oomycosis (caused by the organisms *Pythium inidiosum* and *Lagenidium sp*) and zygomycosis (caused by *Basidiobolus ranarum* and *Conidiobolus coronatus*) are devastating diseases of dogs in the southeastern United States. (Foil, 1984; Grooters, 2000; Hillier, 1994). Both diseases are characterized by chronic granulomatous inflammation of the skin, which results in the development of non-healing wounds and draining tracts. Because the clinical and histologic features of these diseases are similar, they cannot be routinely differentiated from each other on the basis of tissue biopsies. Therefore, patients infected with any one of these four pathogens are often grouped together under the term "phycomycosis" without definitive identification of the causative organism. This is a problem because zygomycetes, as true fungi, are more likely to respond to medical therapy with anti-fungal drugs than are oomycetes, which are not true fungi. Thus there is a need for a specific diagnostic test that can accurately distinguish between infections caused by these organisms.

Immunohistochemical staining of tissue biopsies with polyclonal antibodies has been used extensively for the identification of fungal organisms in animal and human tissues. (Jensen, 1996). However, the production of polyclonal antibodies is laborious and often requires sacrifice of the laboratory animal used for production. The most common host in which antibodies are produced is the rabbit (Hill, 1973; Nerenberg *et al.*, 1978). Rabbits have been used mainly on the basis of their size and docility and the fact that they have been readily available for research purposes (Johnston *et al.*, 1991; Nerenberg, 1978). However, using the rabbit to produce polyclonal antibodies has many drawbacks. Over the past few years, investigators have been searching for a new research animal that is efficient and cost effective.

The domestic chicken has several advantages over the rabbit. One important advantage is reduced cost. At most laboratory animal facilities, the per diem charges for rabbits are at least double those for chickens. According to Rick Ramsey, assistant director of Laboratory Animal Medicine at Louisiana State University, the cost of housing and feeding one rabbit for one day is $0.50, while the cost of feeding and housing one chicken for one day is $0.25. In addition, the initial cost of the animal is much higher for rabbits than for chickens. Louisiana State University is one of many research institutions in the country that no longer breeds rabbits for research purposes due to expense. Rabbits must now be ordered at a cost of $60 per animal. In contrast, the cost of a chicken is only $5.00 per animal. Veterinary costs are also higher for rabbits. A rabbit is governed by the USDA and therefore has strict requirements on living space and temperatures. Chickens can be governed by agricultural standards. They require ambient temperatures and ventilation only, whereas rabbits require an environmental temperature of 68-74°F, usually in an air-conditioned building (Personal communication, Rick Ramsey, 2000).

A second important advantage to using chickens for antibody production is the fact that they produce large amounts of IgY (the chicken equivalent of IgG) in the yolks of their eggs (Larsson *et al.*, 1993). During oogenesis, chickens deposit IgG from their bloodstream into the yolks of their eggs across the follicular epithelium of the ovary (Li *et al.*, 1998; Rose and Orlans, 1981). Once the IgG is accumulated in the yolk, it is more commonly referred to as IgY (Leslie and Clem, 1969; Li *et al.*, 1998). The deposition of IgY in the egg yolk is advantageous goth because of its convenient packaging and its quantity. When rabbits are used to make polyclonal antibodies, they must be bled several times to acquire the necessary amounts of antibodies (Nerenberg *et al.*, 1978). They are usually euthanized and exsanguinated. In contrast, collection of eggs from chickens does not even require handling of the hens, let alone restraint for bleeding.

In addition to being conveniently packaged, the IgY in egg yolks is produced in much larger quantities than those that can be obtained from rabbits. For example, one chicken egg contains almost ten times the amount of antibody that can be obtained from the single bleeding of one rabbit (Bollen and Hau, 1999; Larsson *et al.*, 1993).

A third advantage to using chickens for antibody production is their phylogenetic distance from mammals (Jensenius *et al.*, 1981; Larsson, 1993; Li *et al.*, 1998). Investigators who attempt to produce antibodies against a conserved mammalian protein often find that rabbits and mice have inadequate humoral immune responses to their protein. Chickens, on the other hand, tend to have a more robust immunologic response to mammalian proteins because they are not a mammalian species themselves. For all of the above reasons, we chose to use chickens as hosts in which to raise polyclonal antibodies.

Materials and Methods

Antigen Production

The following isolates were used for production of the antigens: LSU-PI07 (*P. insidiosum* isolated from a dog with intestinal pythiosis); LSU-L01 (Lagenidium sp. isolated from a dog with cutaneous lagenidiosis); ATCC 42064 (*C. coronatus* isolated from human turbinate bone); and ATCC 24671 (B. ranarum isolated from a human with cutaneous basidiobolomycosis). Isolates were incubated in Sabouraud/Dextrose broth containing 5% V8 juice (for *P. insidiosum*), peptone-yeast-glucose broth (for *Lagenidium sp.*), or Sabouraud/Dextrose broth (for *B. ranarum* and *C. coronatus*) at 30°C for five days, killed by the addition of thimerosol (0.02%), and filtered. The mycelial mat was washed three times with

phosphate-buffered saline (PBS), and then ground in the presence of liquid nitrogen. Approximately 200mg of mycelial powder was suspended in 1ml of PBS, vortexed, incubated on ice for 24 hours, and then centrifuged at 10,000 x g for 30 minutes at 4°C. The supernatant containing the soluble mycelial antigens (SMA) was sterilized by passing it through a 0.2 micron filter and then stored at -20°C until used. Protein content of the SMA was determined using the BCA method (BCA Protein Assay; Pierce Chemicals, Rockford, IL).

Antigen Evaluation

Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to evaluate the quality of each mycelial extract prior to its use as an antigen. Protein components of each SMA were electrophoretically separated on a 12% polyacrylamide resolving gel and 4% stacking gel as described by Laemmli (Laemmli, 1970). Briefly, antigen samples were mixed with an equal volume of loading buffer and boiled for 5 minutes at 100°C before application of a 10 ug aliquot to each well. Low molecular weight standards (Bio-Rad Laboratories, Hercules, CA) were also loaded. Samples were subjected to electrophoresis at room temperature for 45 minutes (or until the tracking dye reached the bottom of the gel) and then stained with silver stain (Bio-Rad). Molecular weights of the separated proteins were determined by comparison with the standard.

Chicken Inoculation

Eggs were collected for 7 days prior to inoculation to demonstrate that the birds were not pre-exposed to any of our specific antigens. Antigens were prepared for inoculation as follows. Each of the protein solutions were diluted in PBS to a protein concentration of 1700 ug/ml and

emulsified with equal volumes of Freud's Incomplete Adjuvant (FIA). A total of 0.20 mg of protein was delivered in each of the first two injections, and a total of 0.14 mg of protein (without adjuvant) was delivered in the final injection. Antigen-adjuvant solutions were emulsified using the procedure described by Green and Manson (Green and Manson, 1998). Two leur-lock glass syringes connected by a stopcock were used to make a stable emulsion of antigen and adjuvant. One of the syringes contained the adjuvant and the other contained the antigen. Making sure that the stopcock was open and secure, the mixture was pushed from one syringe to the other until the emulsion thickened.

Hens #1 and #2 were injected with antigens of *P. insidiosum*, hens #3 and #4 were injected with antigens of *Lagenidium sp.*, hens #5 and #6 were injected with *B. ranarum*, and hens #7 and #8 were injected with antigens of *C. coronatus*. Injections were administered via a 19-gauge needle into the pectoralis major muscles on either side of the keel bone. The pectoralis major muscles were chosen as injection sites because chickens possess a renal-portal system. To administer the injection in any other muscle mass would be fruitless because the kidneys would flush out the contents of the injection. The antigen-adjuvant emulsion was divided into two injection sites to decrease the chance of the hens developing abcesses at the sites of injection. The second injection was given 20 days after the first injection, and the final injection was given 40 days after the first injection. Eggs were collected from each hen and labeled for 45 days beginning on the twenty-fifth day after the first injection.

Antibody Extraction

Methods for IgY extraction from egg yolks were modified from those described by Hansen et. al (Hansen et al., 1998). Egg yolks were separated from albumen using a stainless

steel egg separator. Contents of the yolk were extracted using a 14-gauge needle attached to a 12 cc syringe. Yolk extracts from each pair of hens were pooled and then diluted 1:10 in cold 3mM HCl. The pH of the final yolk solution was adjusted to ph=5.0 with 10% acetic acid and this solution was stirred slowly overnight at 4°C. The solution was then centrifuged for 15 minutes at 10,000 x g at 4°C, and the supernatant was collected. Solid ammonium sulfate was added to the supernatant small amounts at a time until 60% saturation (390 g/L) was reached, and this mixture was stirred in the cold for 15 minutes. The mixture was then spun at 4,000 x g for 20 minutes at 4°C to collect the precipitate. The pellet was washed once with cold 60% saturated ammonium sulfate and respun for 10 minutes. The pellet was then dissolved in ten times its volume of PBS. This solution was then referred to as yolk precipitate solution (YPS).

Evaluation of Yolk Precipitate Solution

In order to evaluate the purity of IgY in the yolk precipitate solution, YPS extracted from each pair of hens was evaluated by SDS-PAGE and western immunoblot analysis. Yolk precipitate solution was evaluated using SDS-PAGE analysis as previously described. Proteins on one gel were stained with silver stain (Bio-Rad) for visual analysis. Proteins from a second gel were transferred to a nitrocellulose membrane using a submerged transfer unit (Mini-trans blot; Bio-Rad). The blotted nitrocellulose membrane was blocked for one hour in 5% mild in PBS /0.2% Tween (PBS-T) with 0.02% sodium azide. After washing with PBS-T, the membranes were incubated for one hour with horseradish peroxidase- conjugated rabbit anti-IgY (Rockland Immunochemicals, Gilbertsville, PA) diluted in blocking solution. After extensive washing with PBS-T, membranes were incubated with a chemiluminescent substrate (ECL; Amersham Life Science, Piscataway, NJ). Light signals produced by the enzyme-substrate

reaction were recorded by exposing the membrane to radiographic film (Hyperfilm ECL, Amersham Life Science) for 1-3 minutes.

Evaluation of IgY Specificity

Western immunoblot analysis was used to confirm the specificity of each YPS for its intended pathogen. Protein separation was performed as previously described for SDS-PAGE analysis, with each of the four antigen solutions loaded in sequential wells on each gel. Proteins were then transferred to nitrocellulose membranes. The blotted nitrocellulose membrane was blocked for one hour in blocking solution. After washing with PBS-T, the membranes were incubated for one hour with yolk precipitate containing IgY diluted in blocking solution, washed again for one hour in PBS-T, and then incubated for one hour with the secondary antibody (rabbit anti-chicken peroxidase-labeled IgG; Rockland Immunochemicals) diluted in PBS-T. After extensive washing with PBS-T, membranes were incubated with a chemi-luminescent substrate (ECL; Amersham Life Science). Light signals produced by the enzyme-substrate reaction were recorded by exposing the membrane to radiographic film (Hyperfilm ECL, Amersham Life Science) for 1-3 minutes.

Results and Discussion

The number of eggs produced by each pair of hens was similar (Fig. 1). The average hen lays 5-6 eggs per week (Larsson et al., 1993). As the graph shows, our hens had a much higher average. This is likely due to the fact that our hens were very young. Young adult animals are better antibody producers than older animals (Hanly et al., 1995). Another reason that our hens produced a high volume of eggs with a good antibody concentration is the fact that the birds were housed in a laying house with a controlled environment here at the Louisiana State University Research Farm. Environmental factors influence the laying frequency of chickens (Bollen and Hau, 1999). This is an important factor, especially in states such as Louisiana because the climate is hot and humid.

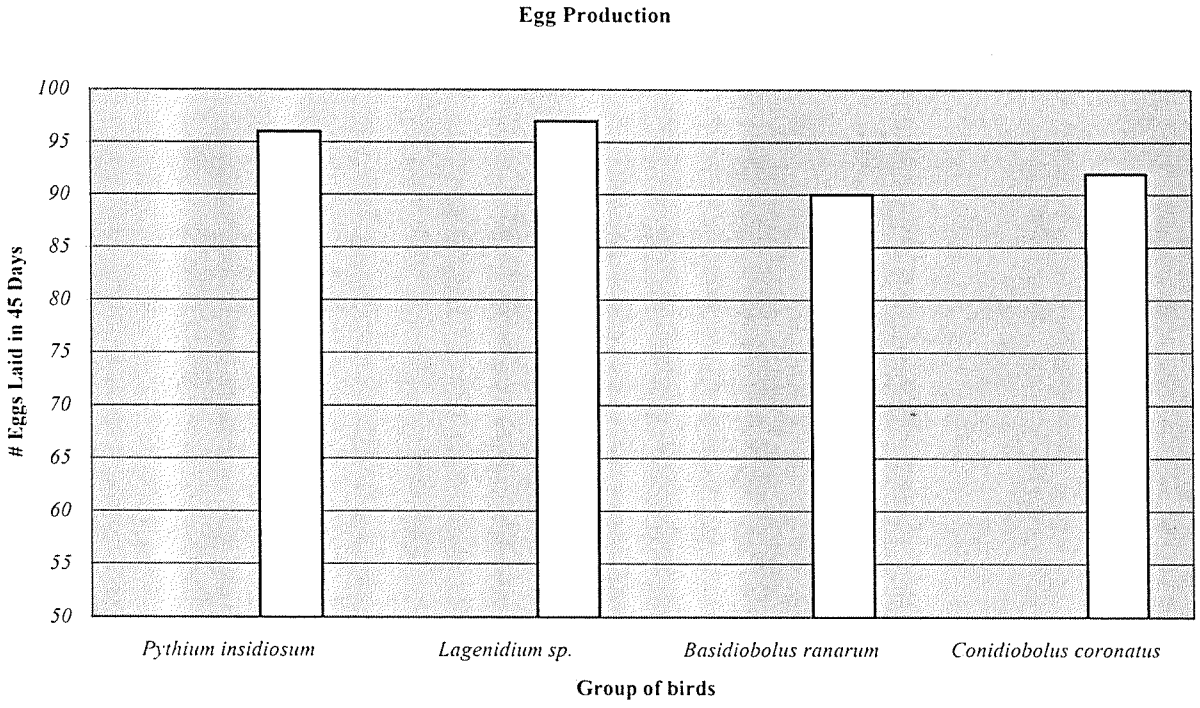

Figure 1

Demonstration of above average egg production in our hens is an important finding because of our choice of adjuvant. Freund's Complete Adjuvant (FCA) is frequently used in polyclonal antibody product because it results in consistently higher titers. Recent research has shown that FCA has a significantly negative effect on egg laying due to the stress that the adjuvant imposes on the hens (Bollen and Hau, 1999). Freund's Complete Adjuvant has been shown to cause focal necrosis, ulceration, fistulous tracts and disseminated granulomas (Bollen and Hau, 1999). Freund's Incomplete Adjuvant has been found to be much safer for the hens (Amyx, 1987; Bennet et al., 1992; Bollen and Hau, 1999; Broderson, 1999; Jackson and Fox, 1995; Jennings, 1995), and it has no negative effects on the laying frequency of hens (Bollen and Hau, 1999). In addition, results of egg and antibody production show that there is no reason to use FCA when using eggs as a source of polyclonal antibodies.

Traditionally, eggs have not been used for polyclonal antibody production due to the difficulty of extracting the antibodies from the yolks of the eggs. In this experiment, improvements were made on IgY extraction techniques. A stainless steel egg separator was used to decrease the contamination of yolk by albumen. Another way of decreasing contamination was to collect the yolk using a 14-gauge needle attached to a 12 cc syringe. For this study, it was decided to use dilution with cold 3 mM HCl as described by Hansen et al (Hansen et al., 1998) to separate the IgY from lipids found in the yolk because it is a simple technique that has been shown to be previously effective.

Evaluation by SDS-PAGE of the YPS produced with this technique showed that there were few contaminants present in the yolk (Fig. 2). In addition, the presence of IgY in the YPS was confirmed by western immunoblot analysis (Fig. 3), indicating that this new technique was effective for IgY extraction.

To evaluate specificity for the intended organisms, yolk precipitate solution from each group of hens was evaluated by western immunoblot analysis. Results showed that antibodies produced in each group of hens were for the most part specific for the pathogen with which the hens were inoculated (Fig. 4). Some cross-reaction was noted between *P. insidiosum* and *Lagenidium sp.*, and also between *B. ranarum* and *C. coronatus*. This is attributed to the fact that *Pythium* and *Lagenidium* are both oomycetes and are therefore very closely related. They share many similar proteins , and that is why antibodies in *Pythium* also recognized antigens in *Lagenidium*. The same relationship is true among *Basidiobolus* and *Conidiobolus*, which are both zygomycetes. The degree of cross-reaction was minimal, and therefore is not expected to be a future problem in the development of the immunohistochemical assay that will be used to diagnose diseases caused by the aforementioned organisms. However, if the cross-reaction does present a problem, adsorption techniques may be used to remove antibodies to the unwanted organisms.

Yolk precipitate solution of pre-immune eggs evaluated by western immunoblot analysis did show a small degree of recognition of antigens of *P. insidiosum* and *Lagenidium sp.* (Fig. 5). This indicates that the hens had previous exposure to an oomycete. One possible hypothesis is that the hens were exposed to *Pythium acanthicum*, an oomycete that is a known pathogen of corn (Vakili, 1985). The reason for this hypothesis is that corn is a main ingredient in chicken feed.

Overall, hens were found to be good hosts in which to raise polyclonal antibodies. They are cost-efficient, docile, and easy to handle. They produce large volumes of eggs with high concentrations of antibodies present in the yolk. Though antibody concentrations within the yolks of the eggs were not specifically quantified in this study, the dilutions made in preparation

for the western immunoblot analysis suggested that the antibody concentrations found in the yolks were approximately ten times higher than the concentrations of antibodies that would have been found in rabbit serum. This estimation is supported by previous findings (Bollen and Hau, 1999). Chickens are also a good host for polyclonal antibody production because of the stability of the antibodies. Some reports indicate that IgY preparations have been stored for up to ten years at 4°C without any loss in antibody activity (Larsson et al., 1994). Finally, chickens are better research animals because they do not require euthanasia at termination of the project nor do they require bleedings that are stressful to the animals and caretakers.

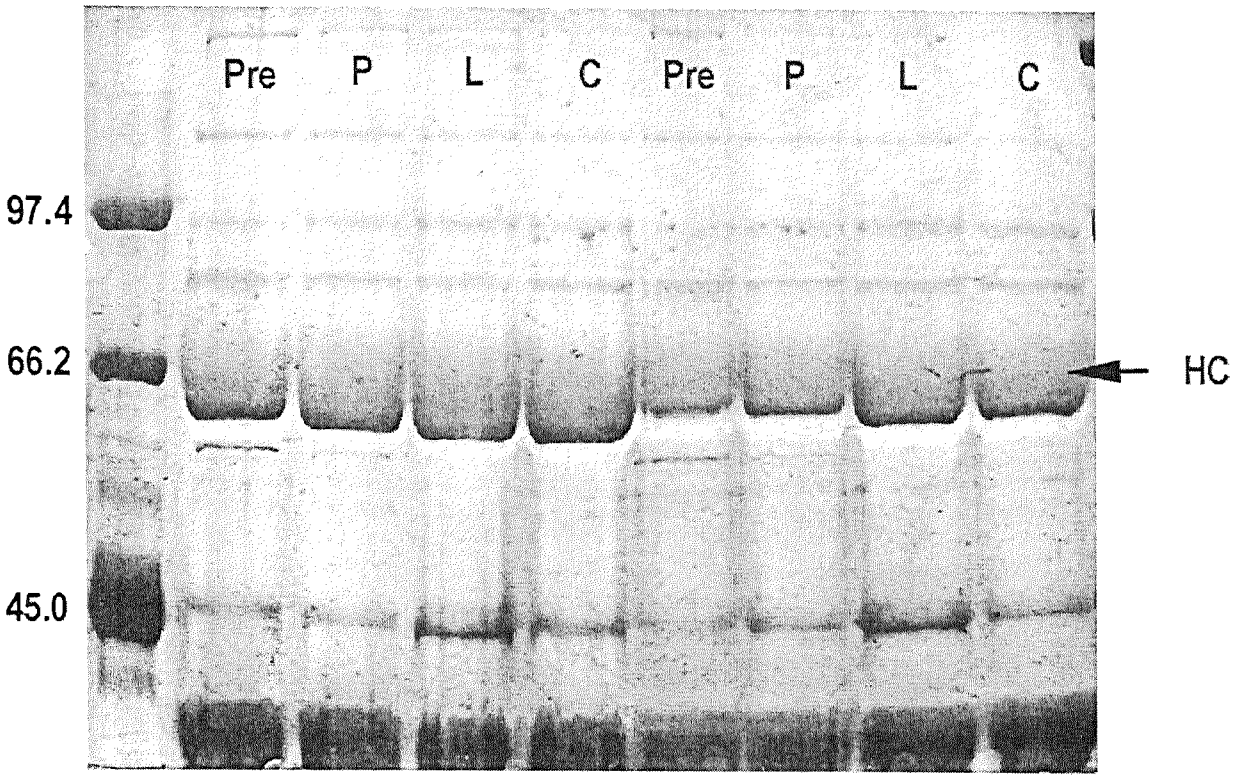

Figure 2: Silver-stained gel showing SDS-PAGE analysis of YPS extracted from pre-immune eggs (Pre), *Pythium* eggs (P), *Lagenidium* eggs (L), and *Conidiobolus* eggs (C). The lane at the left contains molecular weight standards, with sizes indicated in kDa. Lanes 2-5 contain YPS at a 1:3 dilution; lanes 6-9 contain YPS at a 1:10 dilution. The position of the heavy chain fragment is marked (HC).

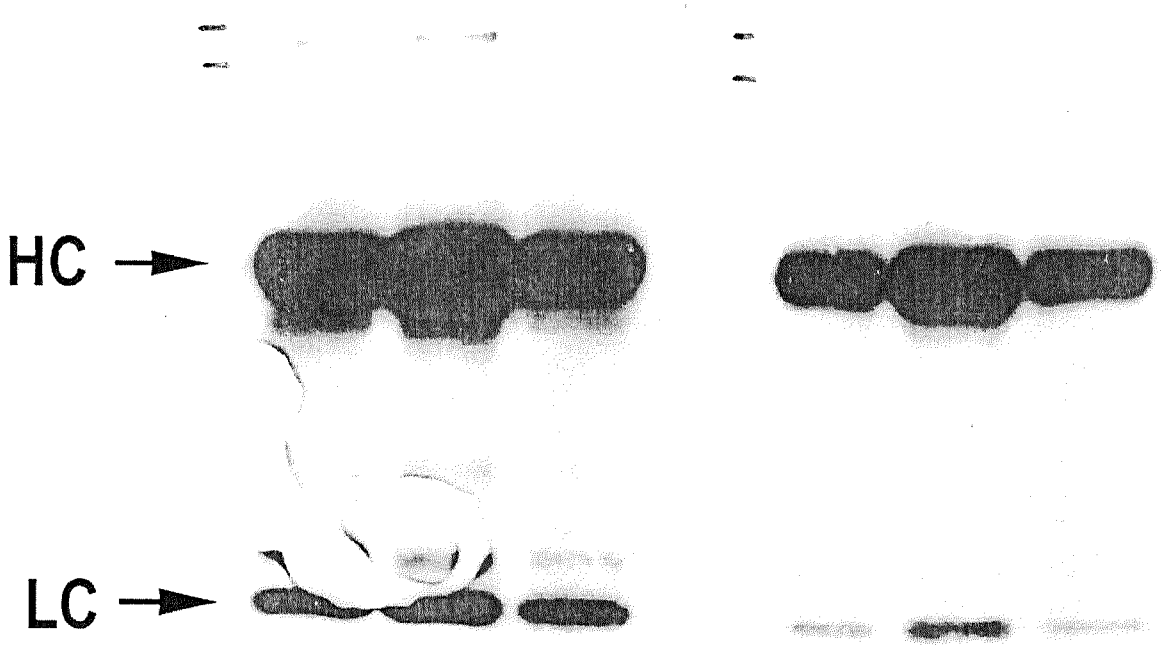

Figure 3: Western immunoblot analysis of YPS showing both the heavy chain (HC) and the light chain (LC) components of IgY. Each lane was loaded with YPS extracted from pre-immune eggs. Lanes 1-3 were probed with HRP-conjugated anti-IgY at a 1:5,000 dilution; lanes 4-6 were probed with HRP-conjugated anti-IgY at a 1:50,000 dilution.

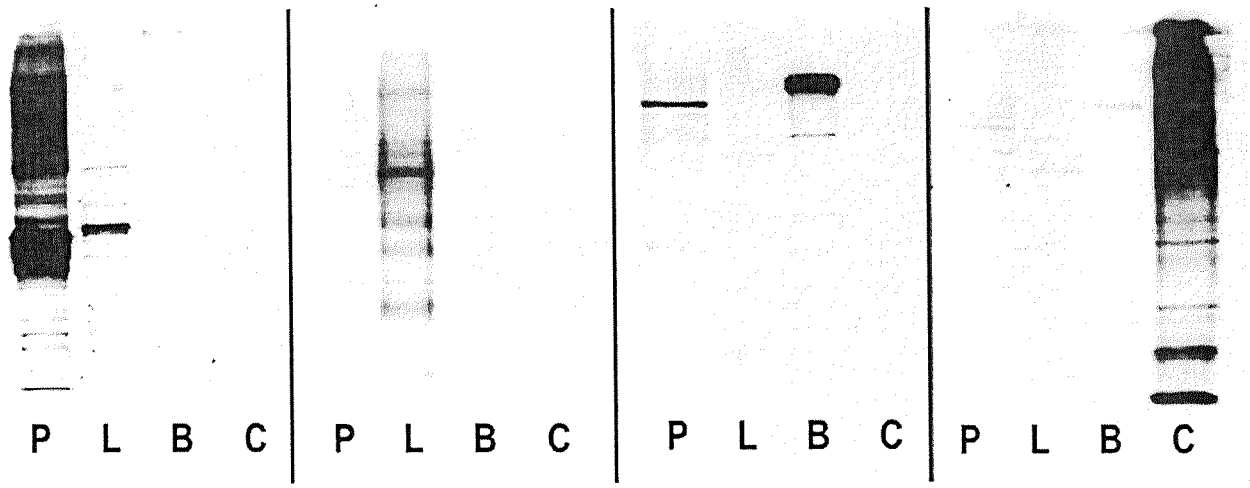

Figure 4: Western immunoblot analysis of polyclonal antibodies raised in chickens against each pathogen. For each immunoblot, lane 1 (P) contains *P. insidiosum* antigens; lane 2 (L) contains *Lagenidium sp.* antigens; lane 3 (B) contains *B. ranarum* antigens; and lane 4 (C) contains *C. coronatus* antigens. The YPS used to probe each blot is listed at the top of the figure.

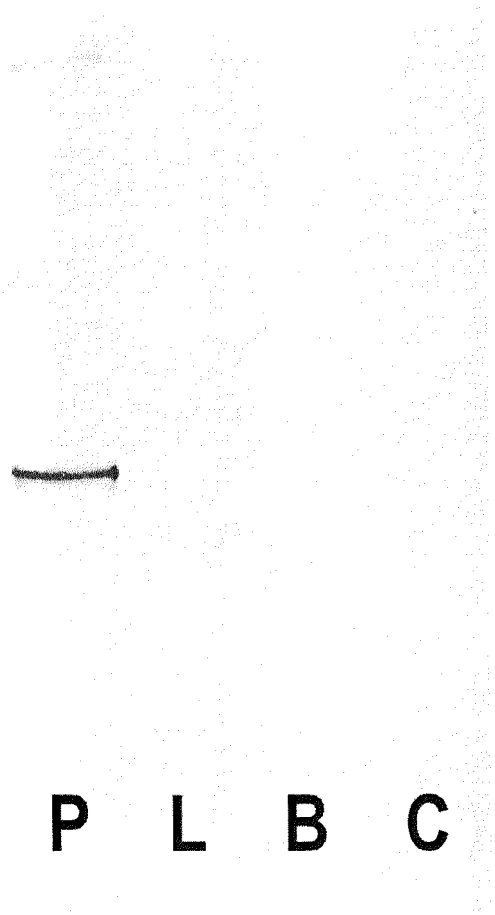

Figure 5: Western immunoblot analysis of YPS from pre-immune eggs.
Lanes are labled as in Figure 4.

Conclusion

Chickens were found to be good producers of polyclonal antibodies. Total volume of eggs collected was above average, and antibody concentration in the yolks of the eggs was high. Improvements made upon antibody extraction techniques were all useful useful in decreasing the amounts of contaminants in the YPS. This procedure was also found to be easier to perform than many of the other antibody extraction procedures. Polyclonal antibodies made in the chicken were found to be specific for the organisms for which the hens were inoculated.

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