Screening Chicken Eggs for the Presence of Anti-*Salmonella* Antibodies

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Abstract

Students work on an investigative project designed around a brief paper obtained from the scientific literature. The project employs readily available materials and entails the screening of eggs purchased from supermarkets, local suppliers and farms for the presence of anti-*Salmonella enteritidis* and anti-*Escherichia coli* lipopolysaccharide antibodies. The presence of anti-bacterial antibodies in eggs can be used as an indirect measure of infection in chicken flocks. Technical precision is required in performing a quantitative ELISA assay, and serial dilutions and the concept of the scientific method are reinforced. As an option, IgY, the major immunoglobulin of chicken, may be purified easily for characterization by standard immunological techniques.

Activity

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INTRODUCTION

Time Required.
As described, the lab work requires one hour during Lab 1, approximately four hours during Lab 2 and one hour during Lab 3. However, if students are given microtiter plates pre-coated with LPS antigen and blocked, the lab work can be completed in one four hour lab period. Also, it is best if the students are given two to three weeks prior to the lab to obtain eggs from commercial and noncommercial sources (if this is impractical, the instructor can provide the eggs). The students should be given the paper by Dadrast et al. (1990) at least one week in advance of the lab work. Data analysis and report writing should take no more than one to two weeks.

Pedagogical Function.
This project provides an opportunity to expose students to an article from the primary literature, to illustrate the scientific method, and to discuss proper experimental design (e.g., the use of controls). Students will participate in an investigative project and gain experience in designing experiments and interpreting data. They also will gain experience in carrying out serial dilutions and performing an ELISA.

Background.
Students should be proficient in working with micropipets and performing and calculating serial dilutions. They should understand the theory behind the ELISA (previous experience in performing an ELISA is desirable but unnecessary).

S. enteritidis infection in poultry flocks is known to be a potential source of human infection. Since infected hens pass anti-*Salmonella* antibodies into their egg yolks, an indirect ELISA may be used to detect the presence of anti-*S. enteritidis* lipopolysaccharide (LPS) antibodies in the eggs of *Salmonella*-infected poultry (as described by Dadrast et al., 1990). IgY is the major class of immunoglobulin in chickens and is analogous to mammalian IgG. In the project described here, you will screen eggs obtained from a variety of sources for the presence of antibacterial (*Salmonella* and *E. coli*) IgYs in chicken egg yolks by ELISA (Goers, 1993). You will coat microtiter plate wells with bacterial LPS (wells coated with dilutions of chicken serum or yolk will serve as a positive control). To the LPS-coated wells you will add serial dilutions of egg yolk obtained from commercial sources and local farms. Finally, rabbit anti-chicken IgG, conjugated to alkaline phosphatase, will be added to detect and titer any antibacterial IgY in the egg yolks.

PROCEDURE

Materials.

- Adjustable or fixed 100 µl micropipetors

- Pipet tips
- Pasteur pipets
- 37°C incubator
- 4°C refrigerator
- Chicken serum [Rockland]
- 0.05 M carbonate buffer (pH 9.6) [Sigma]
- *S. enteritidis* LPS (No. L-6011) [Sigma]
- *E. coli* LPS (No. L-2880) [Sigma]
- Rabbit antichicken IgY alkaline phosphatase conjugate [Promega], diluted 1/5,000 in Superblock [Pierce].
- Eggs - five yolks from each of several sources pooled separately
- TBS (50 mM TRIS, pH 8.0, 27 mM KCl and 138 mM NaCl)
- TBST (TBS containing 0.05% Tween 20)
- Blocking buffer (TBST containing 5% nonfat dried milk)
- Chromogenic substrate (*p*-nitrophenyl phosphate in diethanolamine buffer, at pH 9) [Pierce]
- 96-well polystyrene microtiter plate (note: a microtiter plate reader is optional.)
- If the reagents and supplies listed are used for a class of 20 students, the cost per student is approximately $18.00. One should have enough reagents on hand for 50 student pairs. However, if storred properly (the secondary antibody conjugate must be aliquotted and frozen at -80°C and the other reagents at -20°C or 4°C), I have found that these reagents are good for at least one year.

Student Version.

Lab 1:

1. Assays will be performed in duplicate. Coat all microtiter plate wells in columns 1 and 2 by adding 100 µl of the *S. enteritidis* LPS antigen (100 µg/ml in 0.05 M carbonate buffer, pH 9.6).
2. Repeat step 1 for wells in columns 3 and 4, using a suspension of *E. coli* LPS (100 µg/ml in 0.05 M carbonate buffer, pH 9.6).
3. Seal the microtiter plate by covering the wells with tape or parafilm. Allow the LPS to adsorb overnight, at 4°C. If this timing is not convenient, students may leave the LPS solution in the sealed plates for an extended period (up to 7 days at 4°C, instead of the usual overnight adsorption step).

Lab 2:

1. Remove unbound LPS antigen from the microtiter plate wells by aspiration with a clean Pasteur pipet.
2. Wash the LPS-coated wells three times with TBST. For each wash use a plastic Pasteur pipet to fill each LPS-coated well with TBST, avoiding the formation of large bubbles in the wells. Allow 2 to 3 minutes of soaking in TBST between washes, then "whap out*" the TBST.
(*Next to a sink or basin, place a small stack of paper towels on counter. Quickly invert the microtiter plate, flicking the buffer into the sink. Repeat the flicking, and then remove the last traces of buffer by smacking the plate upside down onto the paper towels.)
3. Block the LPS-coated wells by adding 200 µl of Superblock to each well for 5 minutes.
4. Remove the Superblock by aspiration and add 100 µl of blocking buffer to each LPS-coated well. Each LPS-coated well should now contain 100 µl of blocking buffer.
5. Obtain five eggs from a single source, as described in pre-lab (record the source), and isolate the egg yolks using an egg separator to separate the yolk from the white. Wrap the yolk in cotton gauze; using a wooden applicator or pipet tip, pierce the yolk membrane from the bottom and collect the yolk into a 50 ml tube or beaker.
6. Perform serial 2-fold dilutions of your diluted egg yolk (i.e., the primary antibody) directly in the LPS-coated wells as follows: make a 1/50 dilution of egg yolk in TBS and add 100 µl of the diluted yolk to the 100 µl of blocking buffer already in well A of each of the four LPS-coated columns. Using a clean pipet tip for each column, mix the contents of well A and then transfer 100 µl to well B. Continue to perform 2-fold dilutions through well G (discard the "extra" 100 µl in well G as directed). Repeat this procedure for the other three columns containing LPS-coated wells. You have now added 2-fold dilutions of egg yolk to wells coated previously with bacterial LPS. Note: the wells in row H should be left blank as a negative control.
7. Add 100 µl of TBS to each well in columns 11 and 12. Perform serial 2-fold dilutions in columns 11 and 12. Start by adding 100 µl of a 1/200 dilution of chicken serum to well A and continuing to well G (these wells will serve as a positive control for IgY). Incubate the microtiter plate at 37°C for 1 to 2 hours.
8. Aspirate the fluid from all wells in your microtiter plate with a clean Pasteur pipet and wash the sample-containing wells three times with TBST, as in step 2.
9. Now add to each LPS-coated well and the positive control wells 100 µl of the diluted secondary antibody (a 1/5,000 dilution, in Superblock, of rabbit anti-chicken IgG conjugated to alkaline phosphatase, has been prepared for you). If time permits, continue with step 1 below. Otherwise, seal the plate as before and store at 4°C until the next lab period.

Lab 3:

1. Aspirate the fluid from all wells in your microtiter plate with a clean Pasteur pipet and wash out the secondary antibody, as in step 2 above.
2. Develop the plate to produce a colored product by adding 200 µl of the chromogenic substrate (PNPP) to each well, including wells in row H.
3. After about 30 to 45 minutes, determine which wells are positive by eye, or read the plates at 405 nm using a microtiter plate reader (the wells in row H should remain colorless, but positive wells will turn yellow).
4. For the eggs you used, determine the titer (i.e., the reciprocal of the last positive dilution) of antibacterial LPS antibodies for each bacterial species used. If using a plate reader, plot absorbance at 405 nm against chicken IgY dilutions on semilog graph paper. Record the titer of antibacterial antibodies in the eggs used by different lab groups.
5. Prepare a lab report as described by your instructor.

Safety Issues.

Students will be handling raw eggs and bacterial LPS and should be cautioned to wash their hands and clean up bench tops, etc., carefully with disinfectant. Raw eggs are a potential source of *Salmonella* and other pathogens. In addition to wearing lab coats and proper footwear, students may be advised to wear latex or plastic gloves while isolating egg yolks.

ASSESSMENT and OUTCOMES

Suggestions for Assessment.
Each student should record their ELISA results on the microtiter plate template, and the instructor should inspect their plates. The instructor should look for an appropriate dilution effect, compare the precision of the duplicate columns, and offer suggestions for improvement, as necessary. In evaluating a formal lab report, assessment may be based on the quality of students' data presentation (their own results and the results from other groups should be presented together in a table); the appropriate calculation of dilutions and antibody titers; and the quality of their evaluation of the data (do they effectively compare their results with those of other groups and with those obtained from Dadrast et al., 1990). Their discussion should indicate an understanding of why the presence of antibacterial antibodies is an indication of the bacteria infecting the adult hen, and why these antibodies might benefit the developing chick embryo. Finally, the data should be analyzed with regard to the discovery, or not, of significant titers of anti-*Salmonella* antibodies, the source of any positive eggs, and relevant public health issues.

Problems and Caveats.
In addition to having students prepare ELISA wells in duplicate, it is advisable to have at least two student groups work with the same preparation of egg yolk. We have found relatively high titers of anti-*Salmonella* LPS antibodies in eggs obtained from a few local sources (family-owned farms have been the best source of positive eggs). Conversely, eggs obtained from large commercial suppliers typically do not contain significant titers of anti-*Salmonella* antibodies. Thus, to improve the chances of finding positive eggs, I have students mix five egg yolks together from each individual supplier (one may choose to use fewer eggs). However, the eggs from virtually all sources examined contained high titers of anti-*E. coli* LPS antibodies (thus one should always compare the titers of anti-*Salmonella* and *E. coli* antibodies). I use Pierce's Superblock to block the polystyrene microtiter plate wells, because the recommended blocking time is short (less than 5 minutes). Alternatively, one may use the blocking buffer described above, if the blocking time is extended to 30 to 60 minutes. The isolation of egg yolk is messy and students may need assistance in obtaining the yolks. Finally, in order to reduce the lab work to one four hour lab period, the instructor may choose to give students microtiter plates precoated and blocked with bacterial LPS.

SUPPLEMENTARY MATERIALS

Possible Modifications.
Students may purify the chicken IgY from the yolk and characterize it in a number of ways (i.e., polyacrylamide gel electrophoresis (PAGE), double immunodiffusion, radioimmunoassay, etc.). Western and dot blots against purified bacterial LPS (or bacterial lysates) are a viable alternative to the ELISA described above. One may include other bacterial species. Screening for anti-*Campylobacter* antibodies would be an interesting possibility. I employ a commercial product (Promega's EGGstract) to purify chicken IgY from egg yolks. Other methods for isolating IgY have been described and may be more cost effective, but I have not taken the time to investigate them, as the procedure described here is reliable and works well. The procedure for isolating IgY is given in the appendices.

References.
Dadrast, H., R. Hesketh, and D. J. Taylor. 1990. Egg yolk antibody detection in identification of *Salmonella* infected poultry. *Vet. Record* **126**:219.

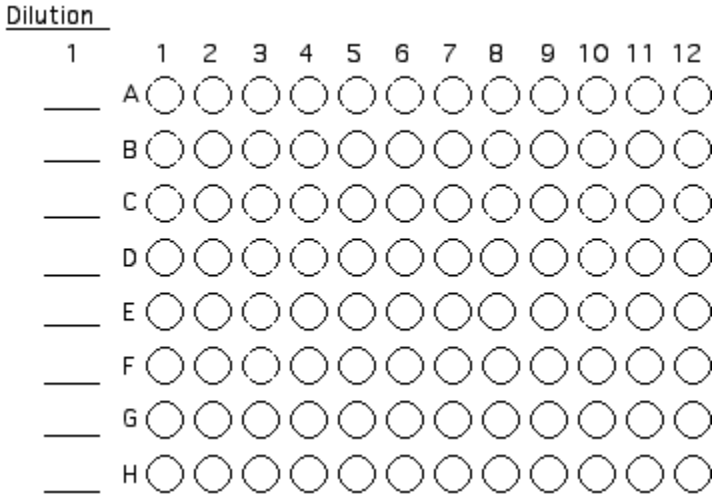
Goers, J. 1993. Immunochemical techniques laboratory manual. Academic Press, Boston, Mass.

Haak-Frendscho, M. 1994. Why IgY? Chicken polyclonal antibody, an appealing alternative. *Promega Notes* **46**:11.

Appendices.
Purification of chicken IgY from egg yolks.
Promega's EGGstract system can be used to purify IgY from egg yolks for about $6.00 per student (yielding over 15 mg of IgY). Promega's procedure is followed, except that the volumes are reduced in order to reduce the per student cost of purifying the IgY.

Wear gloves and wash hands after handling eggs!
1. Allow the eggs to warm to room temperature. Separate the yolk from the white. Rupture the yolk sack with a pipet tip and allow yolk to drip through gauze into a beaker. Transfer 3 ml of egg yolk to a 14 ml graduated polypropylene tube (record source of egg yolk _____).
2. Slowly add 3 volumes (_____ml) of Promega's precipitation solution A (~9 ml) while mixing at room temperature with a wooden applicator stick.
3. After mixing for 5 minutes at room temperature, centrifuge the mixture at 10,000 x g for 10 minutes at 4°C.
4. Collect the supernatant into a graduated tube by filtering it through 4 layers of gauze (discard the pellet). Squeeze the gauze to enhance recovery. Slowly add 1/3 volume (_____ml) of Promega's precipitation solution B, as above.
5. After mixing for 5 minutes at room temperature, centrifuge the mixture at 10,000 x g for 10 minutes at 4°C. Pour off and discard the supernatant.
6. Resuspend the IgY pellet in 3 ml of TBS.
7. An additional precipitation step (repeat steps 4-6) using solution B can be performed to obtain a 90% pure solution of IgY. (A280/1.33 = protein concentration in mg/ml)

ELISA plate template.


Dilution
1
1 2 3 4 5 6 7 8 9 10 11 12
A
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G
H