Detection of Microbial Antibodies in Chickens: Immunofluorescence Studies with Serum and Egg Yolk (34058)

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While searching for simpler and more rapid methods of monitoring chicken flocks for evidence of sarcoma–leukosis (Rous sarcoma and avian leukosis) virus infections, we developed an indirect immunofluorescence test employing egg yolk extract rather than serum (1). Subsequently we found the test to be useful for determining the prevalence of another oncogenic virus infection of chicken flocks; namely, the reticuloendotheliosis (RE) virus (2). This report concerns our studies on the possibility of utilizing the procedure for the detection of other microbial antibodies in birds by examining the yolks of their eggs.

Materials and Methods. Antibody reagents and microbial antigens. Ten 43-week-old White Leghorn hens, purchased from Truslow Farm, Inc., Chestertown, Maryland, were used for experimental immunization. The flock was known to have had an enzootic infection with RIF (resistance-inducing factor) virus.

Prior to immunization all hens were bled and their eggs were collected for antibody titer determinations; thereafter, they were bled at either weekly or biweekly intervals, and the sera were stored until they could be tested simultaneously with the appropriate egg yolk extracts. Eggs were collected daily as available and stored in the refrigerator, but only those eggs laid on or near each bleeding date were used. To prepare the extract, equal volumes of yolk and saline were mixed in a test tube and a double volume of chloroform was added; the tube was inverted several times at 30-min intervals; the saline layer was pipetted off after centrifugation and designated a 1:2 dilution of antibody (1).

Some of the microbial agents used for experimental antibody production were propagated in cell cultures. Primary cultures of chick embryo fibroblasts (CEF), derived from RIF-free, mycoplasma-free eggs (NIH flock), were grown in 32-oz bottles in Eagle's No. 2 medium (3) with antibiotics and other commonly used additives (1). Cell suspensions of a stable cell line MA-104 (rhesus monkey embryonic kidney originated by Microbiological Associates, Inc.) were provided by Tissue Culture Section, Division of Biologics Standards, NIH.

Eastern equine encephalitis (EEE) virus, strain White Filly, was supplied by the Arbovirus Unit of this laboratory as a 20% suspension of infected suckling mouse brain. This was passaged once in CEF cultures; the pooled harvest of culture fluids was used to infect CEF grown on coverslips and also to immunize three of the hens. The coverslip cell cultures were maintained in Falcon plastic petri dishes in a humidified CO_2 (5%) incubator before and after inoculation with 0.1 ml of a virus dilution containing 1000 $TCID_{50}$,[3] as determined by indirect immunofluorescence. When ready for examination, the coverslips were washed in pH 7.2 phosphate-buffered saline (PBS) for several seconds, fixed in cold acetone (—20°) for 10

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[3] Tissue culture 50% infectious doses.

min, air-dried, and either examined immediately or stored at —60° in rubber-stoppered glass shell vials.

A commercial inactivated Rocky Mountain spotted fever (RMSF) rickettsial vaccine was used for repeated immunization of three other hens. The final inoculation of the series employed live *R. rickettsii*, strain Bitterroot, in the form of a yolk sac suspension in Synder's 1 medium (4). A 1:5 dilution in PBS of the pool of the same rickettsia, but grown in BS-C-1 cell culture by Dr. L. F. Barker, Division of Biologics Standards, NIH (5), was used to streak cleaned coverslips for use as antigen in the fluorescence examination.

Each of the other four microbial agents was inoculated without inactivation into one of the remaining hens: Newcastle disease virus (NDV)—strain Kemerovo No. 98; *ornithosis bedsonia*–Texas turkey strain[4]; *Mycoplasma gallisepticum*—strain A-1 (6): and a mixture of *Salmonella pullorum*—strains 17 and 19[5]. The NDV and mycoplasma coverslip preparations employed CEF grown in standard medium; ornithosis bedsoniae were propagated in MA-104 cells in antibiotic-free medium. The salmonellae, grown in broth suspension, were washed with PBS and streaked directly onto cleaned coverslips.

Indirect immunofluorescence procedure. The coverslip preparations were divided into four parts with a special yellow ink (Mark-Tex Corp., Englewood, New Jersey). Each part was flooded with a drop or two of a serial twofold dilution of either serum or yolk extract and the coverslips were incubated at room temperature for 30 min. Details of the staining procedure and the examination for the reactive antibody were described previously (1).

Results and Discussion. To produce demonstrable levels of antibody, the experimental hens were inoculated one or more times with the live or inactivated microbial agents. The antigens were selected as being representative of avian microbial infections important in veterinary and public health; no fungal antigens could be included at this time. Serum and yolk antibody levels against the particular microbial agent were compared by indirect immunofluorescence.

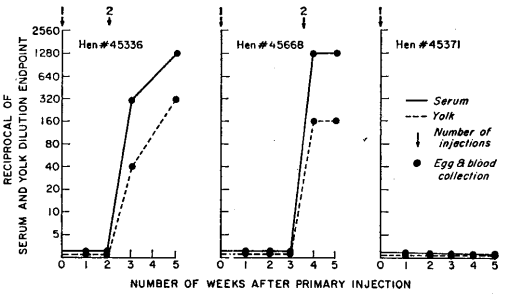

FIG. 1. Immunofluorescence antibody response of three hens to repeated EEE virus injections: comparison of serum and egg yolk titers.

Three hens, No. 45336, 45668, and 45371, were injected subcutaneously with 0.1 ml of an EEE virus dilution in PBS which contained 16 mouse intracerebral LD_{50}[6]. The hens failed to develop serum antibody until after a second injection (Fig. 1). The two hens that received 1000 mouse intracerebral LD_{50} within 1 week developed high serum antibody levels with corresponding but lower egg yolk antibody levels. The third hen was not given a second injection—she did not develop detectable antibody during the 5 weeks of observation.

Each of the three hens in another group, No. 45754, 45304, and 45439, was first inoculated subcutaneously with 0.5 ml of a commercial inactivated RMSF vaccine (Fig. 2). All birds failed to develop detectable antibody; after a second injection of the same vaccine low serum antibody levels were found in two of the birds and none could be detected in the yolk. The antibody was demonstrated in the yolk of the third hen, undoubtedly reflecting a higher serum level. These hens responded with further antibody increase to a third injection of the inactivated vaccine. Finally, when two of the hens were given 5000 egg LD_{50} of living rickettsiae, they responded promptly with much higher serum and yolk levels.

[4] Purchased from the American Type Culture Collection, Rockville, Maryland.

[5] Kindly supplied by Dr. Paul Trainor, University of Maryland.

[6] Fifty per cent lethal doses.

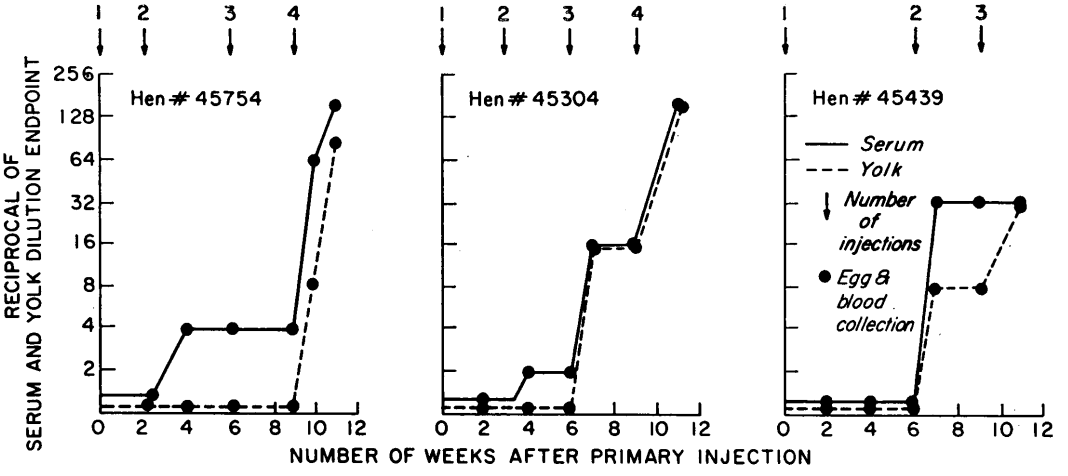

FIG. 2. Immunofluorescence antibody response of three hens to inactivated RMSF vaccine and live RMSF rickettsiae: comparison of serum and egg yolk titers.

The results of these experiments indicated that egg yolk extract probably can be used in place of serum for determining the antibody status of hens after various viral and rickettsial infections. Insofar as the yolk antibody titer tended to be lower than the serum titer, finding of even a barely detectable level of antibody in the egg may be indicative of a significant serum level.

The sera and yolk extracts prepared from the blood and eggs obtained from all ten hens at the beginning of these experiments reacted with NDV, ornithosis agent, *M. gallisepticum,* and *S. pullorum.* It was assumed that this was most likely a result of immunization with NDV and of earlier infection with specific or related salmonella and mycoplasma species; on the other hand, the finding of seemingly specific reactivity with the ornithosis antigen was unexpected. Table I shows that each of the four birds showed a definite serum antibody response 14 days after administration of the respective live antigen with corresponding but lower antibody level in the egg yolk. The one exception was a relatively low level of the antibody to *S. pullorum* in the postinfection yolk sample. Our unpublished immunoelectrophoretic studies (in collaboration with Dr. S. Chaparas, Division of Biologics Standards, NIH) indicated that egg yolk extracts contain almost pure gamma globulin of the IgG type without detectable IgM component; others have reported that injection of enteric gram-negative bacteria into man or rabbit produces a rise in the serum level of IgM, but not of IgG (7, 8).

TABLE I. Immunofluorescence Antibody Levels in Sera and Eggs from Hens after Administration of Live Antigens.[a, b]

Antigen	Pre[c]		Post[c]	
	Serum	Egg	Serum	Egg
Newcastle disease virus	40	40	640	160
Bedsonia (ornithosis)	20	10	640	320
M. gallisepticum	40	20	1280	320
S. pullorum	10	10	320	20

[a] By indirect immunofluorescence test.

[b] Sera and eggs collected 14 days after intraperitoneal injection of each live antigen.

[c] Results expressed as the reciprocal of serum and egg dilution endpoints.

The fluorescence patterns seen with each of the antigens stained with yolk were similar to those observed with serum-stained antigens. The distinctive appearance of the ornithosis bedsonia in the indirect immunofluorescence test to our knowledge has not been recorded in the literature: Fig. 3 shows a typical preparation—the pattern was the same whether serum or egg yolk served as the source of antibody.

The use of egg yolk rather than serum

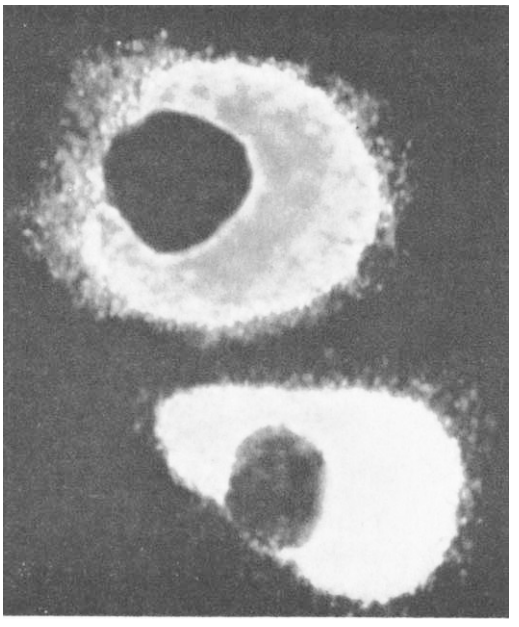

FIG. 3. Characteristic appearance of the ornithosis bedsonia-infected MA-104 cell in the indirect immunofluroescence procedure.

eliminates potentially life-threatening trauma and infection of the birds and saves the investigator's time and effort. Even though the question of immunologic specificity of the fluorescence antibody in serum and in egg has not yet been settled, this simple, rapid, and inexpensive procedure should be useful in monitoring the microbial antibody status of chicken flocks, as well as wild bird populations.

Summary. The indirect immunofluorescence test, employing egg yolk extract rather than hen's serum, should offer an important additional tool for the detection of certain microbial antibodies in domestic and wild birds.

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