

## VIREMIA AND SEROLOGICAL RESPONSES IN ADULT CHICKENS INFECTED WITH WESTERN EQUINE ENCEPHALOMYELITIS AND ST. LOUIS ENCEPHALITIS VIRUSES<sup>1,2</sup>

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**ABSTRACT.** Adult hens, similar to those used for arbovirus surveillance, were experimentally infected with western equine encephalomyelitis (WEE) and St. Louis encephalitis (SLE) viruses to describe the viremia response, to compare serological testing methods, and to evaluate a new method of collecting whole blood onto filter paper strips from lancet pricks of the chicken comb. Young (19 weeks), but not old (38 weeks), hens developed a low-titer, transient viremia for a 1-day period. Immunoglobulin G (IgG) was detected by days 10 and 14 after infection with WEE and SLE viruses, respectively, by indirect fluorescent antibody tests, hemagglutination inhibition tests, and plaque reduction neutralization tests on sera and in direct enzyme immunoassays (EIA) on both sera and eluates from filter paper samples. Immunoglobulin M (IgM) was first detected in sera 2 and 3 days before IgG, respectively, but IgM could not be detected reliably in eluates from dried blood. Sera and dried blood samples collected from naturally infected sentinel chickens gave comparable results when tested by an EIA for IgG.

### INTRODUCTION

Western equine encephalomyelitis (WEE) and St. Louis encephalitis (SLE) viruses are zoonoses maintained in western North America by horizontal transmission between *Culex tarsalis* Coq. mosquitoes and passeriform birds (McLean and Bowen 1980, Hardy 1987, Reisen and Monath 1989, Reeves 1990). Chickens frequently become infected during summer concurrently with virus amplification in the passeriform bird population and have been recognized as effective sentinels (Reeves et al. 1990). Sentinel chickens provide a more sensitive and cost effective indicator of low to moderate virus activity than does testing pools of *Culex* mosquitoes for virus infection (Reisen et al. 1992a, 1992b) and, if sampled frequently, may provide an early warning of human infection (Reeves and Hammon 1962, Reisen et al. 1992c). In comparison with sampling wild bird populations, sentinel chickens simplify laboratory testing and provide more

specific information on the time and place of virus transmission.

Currently, the California Encephalitis Virus Surveillance (EVS) Program relies primarily on seroconversions in sentinel chickens to detect enzootic virus amplification. In recent years, flocks of 10 adult white leghorn laying hens have been deployed at more than 140 locations statewide (Emmons et al. 1993). Prior to 1993, hens were bled monthly or biweekly from April to October by jugular venipuncture by mosquito control or public health agency personnel, and the sera was sent either refrigerated or frozen to the Viral and Rickettsial Disease Laboratory in Berkeley, CA, for testing with an enzyme immunoassay (EIA) that detected a rise in specific immunoglobulin G (IgG) antibody.

Although chickens are widely used as sentinels, little is known about the interval required from infection with WEE and SLE viruses until the detection of serum antibodies in adult chickens (> 18 wk of age), especially using newly developed EIAs. Knowledge of this interval is critical in estimating when the seropositive sentinel was bitten by an infected mosquito. Sudia and Chamberlain (1959) found that hemagglutination inhibition (HI) and neutralizing antibodies to SLE virus appeared within 2 wk post-infection (PI) in 12-wk-old chickens. LaMotte et al. (1967) conducted similar studies with both WEE and SLE viruses and reported that most adult birds seronegative 1 wk PI became seropositive by 2 wk PI. Recently, Olson et al. (1991) found that 4-wk-old chickens experimentally infected with eastern equine encephalomyelitis virus developed antibodies detectable by HI test, plaque reduction neutralization test (PRNT), and EIAs to IgG and immunoglobulin M (IgM) antibodies

<sup>1</sup> An interim unrefereed summary of this research was reported previously (Reisen et al. 1993a).

<sup>2</sup> Protocols for the care and use of vertebrate animals in this research were described in Animal Use Protocols R009-0592B and -0693B "Arbovirology Ecology and Vector Competence Studies" approved by the Animal Care and Use Committee of the University of California, Berkeley.

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within 2–4 days PI. Unfortunately, comparable data are not available for adult chickens infected with WEE and SLE viruses. These data are useful because in California sentinel chickens are deployed when  $\approx 18$  wk old and repeatedly bled until  $>40$  wk of age. Therefore, one purpose of the present research was to determine the interval from virus infection to antibody rise in adult chickens experimentally infected with WEE and SLE viruses. Because sentinel chickens frequently are placed near human residences in periurban habitat, we also described the viremia response in adult chickens to determine if they could serve as a source of infection for mosquitoes.

Using a centralized laboratory to test sentinel chicken sera assures quality control and comparability of results but may result in delays when sera must be shipped refrigerated or frozen by ground transport and cannot be received on weekends. Antibodies can be detected readily in whole human blood spotted onto paper strips, and this method is used routinely by the California Department of Health Services for screening human blood for antibodies to viruses such as human immunodeficiency virus. Use of a similar method for sentinel chickens would expedite the collection and shipment of blood samples to the laboratory. Therefore, a second purpose of the present research was to develop a simple method of bleeding sentinel chickens that would allow the rapid collection and shipment of blood specimens to the laboratory without sacrificing current sensitivity or specificity of serological tests for WEE and SLE antibodies.

## MATERIALS AND METHODS

**Chickens:** Adult white leghorn laying hens were procured from Eggs West in Victorville, CA, and were transported either to Bakersfield for experimental infection or to sentinel flock cages in southeastern California. Birds were wing-banded and pre-bled prior to experimental infection (day 0, Table 1) or deployment as sentinels. All sera were negative for antibodies to WEE and SLE viruses at this time.

**Experimental infection:** Groups of 5 hens were inoculated subcutaneously with either  $4.3 \log_{10}$  plaque forming units (PFU) of WEE virus (BFS 1703) or  $3.2$  and  $4.1 \log_{10}$  PFU of SLE virus (BFS 1750). Both WEE and SLE virus strains were isolated originally from *Cx. tarsalis* mosquitoes collected in Bakersfield in 1953 and had been passaged twice in suckling mice by intracranial inoculation. A control hen was inoculated with diluent (without virus) and placed in the same cage with each treatment group.

**Blood collection:** On days 1–10 PI, 0.5 ml of

whole blood was withdrawn from each hen by jugular venipuncture and frozen at  $-70^{\circ}\text{C}$  for later viral assay. On alternate days for 18 days, weekly for the next 4 wk, and then biweekly for 12 wk PI, 5–7 ml of blood was collected by jugular venipuncture, centrifuged, and the sera frozen for later assay. Concurrently on days 0–35 PI, a 0.75-in.- (1.9 cm) long portion of a  $0.5 \times 3.0$ -in. ( $1.3 \times 7.6$  cm) Whatman No. 1 filter paper strip was soaked with blood, allowed to air dry under ambient conditions, stapled to a  $5 \times 8$ -in. ( $13.0 \times 33.8$  cm) index card, placed into a plastic bag, and then stored in a refrigerator until later tested for antibody.

**Virus assays:** Samples of whole blood were titrated for infectious virus by plaque assay in Vero cell culture (Reisen et al. 1993b). Blood from hens inoculated with WEE virus was triturated in mosquito diluent (20% fetal bovine serum [FBS] in phosphate-buffered saline [PBS] containing 100 units penicillin, 200  $\mu\text{g}$  streptomycin, 100  $\mu\text{g}$  gentamycin, and 100 units nystatin), whereas blood from hens inoculated with SLE virus was triturated in 0.75% bovine albumin (BA) in borate buffered saline (pH 9.0) containing the same antibiotics. Virus in serial dilutions of triturates was allowed to absorb in Vero cell cultures for 1.5 h at  $36^{\circ}\text{C}$ , rinsed with PBS, overlaid with agar, and then incubated at  $36^{\circ}\text{C}$ . Plaques were counted at 4 (WEE virus) or 12 (SLE virus) days. Cultures infected with WEE virus received a single overlay with Oxoid agar in minimal essential media (MEM) containing FBS, DEAE dextran, antibiotics, and neutral red. Overlay medium for cultures infected with SLE virus was similar but did not contain neutral red, thus requiring a second overlay with neutral red on days 3–4 PI.

**Antibody assays:** Indirect EIAs for specific IgG in chicken sera were performed using a modification of the method described by Calisher et al. (1986). Briefly, wells of 96-well plates were coated overnight at  $4^{\circ}\text{C}$  with antigen prepared from Vero cell cultures infected with WEE (BFS 1703) or SLE (BFS 1750 or Ruis) viruses or “mock infected” as a negative control diluted in carbonate-bicarbonate coating buffer (pH 9.0). Wells were washed with PBS-Tween 20 (PBS-T) and then blocked with 10% horse serum in PBS-T and incubated at  $37^{\circ}\text{C}$  (as were all subsequent steps) for 60 min. Chicken serum was diluted 1:100 in PBS-T with 0.5% BA and added to the wells. After the plates were rinsed with PBS-T, biotinylated goat anti-chicken IgG conjugate (Vector Laboratories, Burlingame, CA) diluted in PBS-T with 0.1% BA was added and the plates were incubated for 30 min. After washing with PBS-T, the wells were treated with a peroxidase-labeled avidin-biotin complex (Vecta Stain, Vec-

Table 1. Serological response of 19- and 38-wk-old hens (*n*, 5 per trial) on days 0–21 post-infection with western equine encephalomyelitis (WEE) or St. Louis encephalitis (SLE) viruses (viral dose in parentheses).

Days post-infection	Serological test <sup>1</sup> (No. positive [OD or GMT])					
	IgG EIA		IgM EIA	HI	PRNT	IFA
	BS	S				
WEE virus (4.3 log <sub>10</sub> PFU)						
0	0 (0.3)	0 (0.2)	0 (0.5)	0 (<20)	0 (<20)	0
2	0 (0.3)	0 (0.2)	0 (0.7)	0 (<20)	0 (<20)	0
4	0 (0.3)	0 (0.2)	0 (0.7)	0 (<20)	0 (<20)	0
6	0 (0.9)	0 (0.5)	4 (1.8)	0 (<20)	0 (<20)	0
8	4 (2.6)	4 (1.6)	5 (2.8)	5 (35)	2 (20)	4
10	5 (3.5)	5 (2.8)	5 (2.3)	5 (53)	5 (61)	5
12	5 (3.6)	5 (3.2)	5 (1.9)	5 (121)	4 (94)	5
14	5 (3.5)	5 (3.0)	5 (2.1)	5 (114)	4 (79)	5
16	5 (2.9)	5 (2.6)	5 (1.9)	5 (70)	5 (40)	5
18	5 (2.9)	5 (2.8)	5 (2.3)	5 (61)	5 (53)	5
21	5 (3.1)	5 (2.7)	5 (2.3)	5 (53)	5 (121)	5
SLE virus (3.2 log <sub>10</sub> PFU)						
0	0 (0.4)	0 (<0.1)	0 (0.5)	0 (<20)	0 (<20)	0
2	0 (0.4)	0 (<0.1)	0 (0.6)	0 (<20)	0 (<20)	0
4	0 (0.6)	0 (<0.1)	0 (0.6)	0 (<20)	0 (<20)	0
6	0 (0.3)	0 (0.1)	1 (0.8)	0 (<20)	0 (<20)	0
8	0 (0.3)	0 (0.1)	2 (0.9)	0 (<20)	0 (<20)	0
10	0 (0.4)	2 (1.0)	3 (1.4)	0 (<20)	0 (<20)	0
12	3 (2.0)	3 (2.5)	3 (2.1)	3 (20)	4 (40)	3
14	5 (2.2)	5 (3.0)	4 (2.4)	4 (24)	4 (56)	3
16	5 (2.5)	4 (3.3)	4 (2.4)	5 (26)	4 (56)	4
18	5 (2.9)	4 (3.2)	4 (2.1)	5 (30)	5 (46)	4
21	5 (2.4)	5 (2.7)	4 (3.1)	5 (40)	5 (46)	4
SLE virus (4.1 log <sub>10</sub> PFU)						
0	0 (0.6)	0 (<0.1)	0 (0.5)	0 (<20)	0 (<20)	0
2	0 (0.4)	0 (<0.1)	0 (0.4)	0 (<20)	0 (<20)	0
4	0 (0.4)	0 (<0.1)	0 (0.6)	0 (<20)	0 (<20)	0
6	0 (0.7)	0 (<0.1)	0 (0.5)	0 (<20)	0 (<20)	0
8	0 (0.4)	0 (<0.1)	1 (1.0)	0 (<20)	0 (<20)	0
10	0 (0.6)	5 (1.5)	5 (2.0)	4 (20)	2 (20)	1
12	1 (1.5)	5 (3.5)	5 (2.8)	5 (40)	5 (53)	4
14	5 (2.3)	5 (3.7)	5 (2.0)	5 (23)	4 (56)	5
16	5 (2.4)	5 (3.7)	5 (1.7)	5 (23)	3 (79)	5
18	5 (2.2)	5 (3.8)	4 (1.5)	5 (20)	3 (40)	5
21	5 (2.2)	5 (3.9)	5 (3.8)	5 (20)	3 (63)	5

<sup>1</sup> Serological tests: IgG EIA, enzyme immunoassay testing for immunoglobulin G, IgG, in blood spotted on filter paper, BS, or in sera collected by venipuncture, S; IgM EIA, enzyme immunoassay testing for immunoglobulin M, IgM; HI, hemagglutination inhibition test; PRNT, plaque reduction neutralization test using Vero cells; IFA, indirect fluorescent antibody test. For EIAs subtractive optical densities, OD, are averaged for all hens, whereas for the HIs and PRNTs the reciprocal geometric mean titers, GMT, were averaged only for seropositive hens.

tor Laboratories) and incubated for 30 min. Plates were washed with PBS and the substrate, ABTS (Kirkegaard and Perry Laboratories, Gaithersburg, MD), was added. Plates then were incubated for 15–20 min and read at 405 nm. For

EIAs, values reported were the subtractive optical densities corrected by the negative controls run on each plate. Optical densities were considered positive when they exceeded the mean of the positive and negative controls for each plate.

Similar EIAs for IgG were done on eluates of whole blood from the filter paper strips. Two punches from each filter paper strip were placed into one well of a 96-well plate containing 200  $\mu$ l of PBS-T with 0.5% BA. Samples were held at room temperature for 30 min and then at 4°C overnight. The next day, 50  $\mu$ l of eluate and 50  $\mu$ l of diluent (final dilution  $\approx$  1:44) were added to each antigen-coated test well, previously blocked with 2.0% casein in PBS-T for 2 h at 37°C. Tests on all sera and eluates by IgG EIA were confirmed by indirect fluorescent antibody assay (IFA).

The indirect EIA for IgM was performed similar to the EIA for IgG, except that sera were diluted 1:80 prior to assay and biotinylated goat anti-chicken IgM was used. Commercially prepared goat anti-chicken IgM (ICN Biochemical, Irvine, CA) was purified by dialysis against borate-buffered saline (pH 8.0). Biotinylation was performed with a biotin-n-hydroxysuccinimide ester (Behring Diagnostics, La Jolla, CA) in dimethyl sulfoxide, followed by dialysis against PBS (pH 7.2) and passage through a Sephadex G24 column.

Hemagglutination inhibition tests used goose erythrocytes, sucrose-extracted WEE (BFS 1703) and SLE (BFS 2108) virus hemagglutinins from infected mouse brains, and acetone-extracted sera according to the methods of Clarke and Casals (1958).

The PRNT previously described for bunyaviruses by Campbell et al. (1989) was modified using the media and incubation times described above for the plaque assay of WEE and SLE viruses. Serial 2-fold dilutions of sera were incubated with challenge doses of  $\approx$  85 PFU of either WEE or SLE virus. Unneutralized virus was detected by plaque assay. An 80% reduction in the number of plaques was considered positive.

**Field evaluation:** Six flocks of 10 adult sentinel hens were exposed in southeastern California and were bled concurrently by lancet prick of the comb and by jugular venipuncture at bi-weekly (3 sites, Coachella Valley) or monthly (3 sites, Imperial Valley) intervals from April through October 1992. Sentinel chickens that seroconverted were replaced with seronegative birds after a confirmatory bleed. Samples of whole blood absorbed from the comb onto the filter paper strips were dried and stored under ambient conditions. Sera were shipped to the laboratory on dry ice and tested immediately for IgG against WEE and SLE viruses using appropriate EIAs. Although no formal attempt was made to conduct a blind evaluation, filter paper samples were tested by EIA independently at a later date by

J.L., who did not have immediate access to sera sample test results.

## RESULTS

### Experimental infections

**Viremia:** Of 5 hens inoculated with WEE virus at 19 wk of age, 3 had detectable viremias (2.3, 2.3, and 2.6  $\log_{10}$  PFU/0.1 ml) only on day 2 PI, and one had a viremia (2.6  $\log_{10}$  PFU/0.1 ml) only on day 3 PI; the remaining hen was negative throughout. None of the 38-wk-old hens inoculated with SLE virus and none of the 3 control hens developed a detectable viremia.

**Serology:** EIAs on sera from hens infected with WEE virus were first positive for IgM (mean, 6.4 days; range, 6–8 days) 2 days earlier than for IgG (8.4 days; range, 8–10 days) (Table 1). Results of IFA and HI tests on sera and IgG EIAs on filter paper eluates were similar to the IgG EIAs done on sera. Antibodies did not effectively inhibit plaque formation until slightly later (PRNT mean, 9.2 days; range, 8–10 days) and results from one hen initially were variable; i.e., titers were <1:20 on days 2–6, 1:20 on days 8 and 10, <1:20 on days 12 and 14, and then  $\geq$  1:20 for days 16–126, peaking at 1:160 on day 70 PI. Serum IgM EIAs and filter paper IgG EIAs were positive through day 35 PI, whereas the remaining tests were positive through day 126 PI (last day tested by each method).

Four of 5 38-wk-old hens inoculated with 3.2  $\log_{10}$  PFU of SLE virus developed antibodies detectable by all assays (Table 1). The remaining hen was positive by HI and PRNT tests on sera and IgG EIA on filter paper eluates but variable by IgG EIA on sera and negative by IgM EIA and IFA on sera. Similar to WEE virus, tests done on sera were first positive by IgM EIA ( $n$ , 4 hens; mean, 9.5 days; range, 6–14 days), 2.5 days earlier than by IgG EIA ( $n$ , 5; mean, 12.0 days; range, 10–14 days) and 3–4 days earlier than HI (mean, 13.2 days), PRNT (mean, 12.8 days), IFA (mean, 13.0 days), and filter paper IgG EIA (mean, 12.8 days). Two hens gave variable PRNT results on days 12–35 but then were consistently positive until last bled on day 126 PI. Hens were positive by the remaining tests throughout the remainder of the experiment.

When inoculated with 4.1  $\log_{10}$  PFU of SLE virus, 38-wk-old hens produced a more rapid and uniform antibody response than when inoculated with 3.2  $\log_{10}$  PFU (Table 1). All 5 hens developed antibodies detectable by all tests. Sera tested by IgM EIA were positive first (mean, 9.6 days), followed by IgG EIA (10.0 days), HI (10.4 days), PRNT (11.2 days), IFA (12.0 days), and IgG EIA on filter paper eluate (13.6 days). Three

of 5 hens initially exhibited variable PRNT results, but all were consistently positive from days 35 to 126 PI. Hens remained positive by all tests throughout the remainder of the experiment.

Control birds in all groups remained antibody negative by all tests for the duration of the experiments, indicating that WEE or SLE virus was not transmitted by direct contact among birds in the same cage.

#### Field evaluation of filter paper methods

A total of 461 sera and filter paper blood samples from sentinel hens exposed in southern California during 1992 were tested for WEE and SLE viruses by IgG EIAs. Overall, 34 serum and 32 filter paper samples were positive for WEE antibodies and 38 and 37 for SLE antibodies, respectively. There were no false positives. Three hens were positive for both WEE and SLE antibodies by assays on both samples. The 3 negative filter paper samples collected from hens positive by sera EIA had optical density readings that were just below the conservative cut-off value. Because filter paper samples from experimentally infected hens frequently were first positive >1 day after serum samples, these 3 hens were presumed to be infected but were sampled before antibody titers were sufficiently elevated to give a positive test.

#### DISCUSSION

An arbovirus sentinel should not be a source of virus infection for mosquitoes, especially if deployed in residential habitats. When tested by plaque assay in Vero cells, 19-wk-old hens produced a transient, low-titer ( $2.3\text{--}2.6 \log_{10}$  PFU/0.1 ml) viremia to WEE virus. Four of 5 19-wk-old hens concurrently infected with low dose of SLE virus ( $2.9 \log_{10}$  PFU) also produced a transient, low-titer viremia ( $<2.4 \log_{10}$  PFU/0.1 ml) of less than 1 day duration (data not shown). In contrast, all 38-wk-old hens infected with higher doses of SLE virus ( $3.2$  or  $4.1 \log_{10}$  PFU) failed to produce a detectable viremia. These results differed somewhat from previous studies that assayed sera for virus using mice inoculated intracranially. Hammon and Reeves (1946) reported that sera from all adult hens experimentally infected with WEE virus killed mice from 18 to 64 h PI, but viremia titers were not determined. Sudia and Chamberlain (1959) reported that 12-wk-old chickens infected with SLE virus produced sufficient viremia to kill mice on days 3–7 PI. LaMotte et al. (1967) found that adult chickens infected with WEE or SLE viruses produced sufficient viremias to kill mice 1–2 days and 1–6 days PI, respectively. Viremia titers detected by Vero cells in the present study generally

were low and probably were sufficient to infect only the most susceptible *Culex* mosquitoes (Hardy and Reeves 1990). During mid-summer, the median dose of WEE and SLE viruses required to infect *Cx. tarsalis* in Kern County was found to range from 2.5 to 5.7 and from 2.5 to  $6.1 \log_{10}$  PFU/0.03 ml, respectively (Hardy et al. 1990). Therefore, sentinel hens would have the greatest risk as a source of virus to infect mosquitoes when first deployed in the spring, usually when 18–22 wk old. By mid-July, when virus activity usually is first detected in California, hens would be >30 wk old and should no longer serve as a source of virus infection for mosquitoes.

In a large surveillance program, serological assays should be specific, sensitive, and cost effective as well as provide the earliest possible detection of infection. IgM antibodies to both WEE and SLE virus were detectable  $\approx 2$  and 3 days earlier than were IgG antibodies, respectively; however, biotinylated anti-chicken IgM is not commercially available, an important consideration in a laboratory that performs EIAs on >1,000 hens bled biweekly for a 6–8-month period. In addition, IgM antibodies were not detected successfully in eluates from blood samples dried onto filter paper, despite the use of Tween in the diluent. In contrast, all tests to detect IgG antibodies produced similar results, except for the single 38-wk-old hen inoculated with  $3.2 \log_{10}$  PFU of SLE virus that was negative by serum IgM and IgG EIA but positive by serum HI and PRNT and by eluate IgG EIA. Hens were positive by all tests for IgG antibodies to WEE virus 10 days PI and to SLE virus after 14 days PI. However, depending on the serological test, 38-wk-old hens inoculated with  $4.1 \log_{10}$  PFU of SLE virus developed detectable antibodies 0.5–3.5 days earlier than did hens inoculated with  $3.2 \log_{10}$  PFU. These data indicated that the length of the interval between infection and antibody detection may depend upon the quantity of virus delivered by the mosquito.

The ease of specimen collection and processing is critical to the acceptability of a large-scale and widely dispersed sentinel program that relies upon collaborating agencies to collect blood specimens. Prior to 1993, sentinel hens were bled by jugular venipuncture using vacutainers, a technique requiring some training and manual dexterity. Blood then had to be centrifuged, the sera decanted, and shipped to the laboratory either refrigerated or frozen. Needles and blood products then had to be disposed of at a medical waste facility. The new bleeding method evaluated in the present study was relatively simple to perform, inflicted minimal trauma to the sentinel hens, required minimal specimen handling, and produced little medical waste. Specimens can be

shipped via overnight mail to the laboratory on any day of the week, greatly expediting testing and precluding sample loss due to inadvertent thawing. In practice, sentinels may be bled and the filter paper strips mailed to the laboratory on Monday, received and eluted on Tuesday, and tested by EIA on Wednesday. Positives can be confirmed by IFA on Thursday and the results reported by phone or fax on Thursday or Friday. Therefore, with a biweekly bleeding schedule, the time from infection by mosquito bite to detection of antibodies would range from 15 to 28 days for WEE virus and from 19 to 32 days for SLE virus, presuming that some infected hens were bled before they developed detectable IgG. Tests on whole blood collected by our new bleeding method were equally sensitive in detecting IgG antibodies in both experimentally and naturally infected adult hens. Starting in 1993, the California EVS Program has adopted this new bleeding method for the sentinel chicken program. Results in 1993 indicate that the new bleeding method was well accepted by both field and laboratory personnel.

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