Justification for the proposed changes to the current 9 CFR 94.6 regulations governing the importation of table eggs from regions where exotic Newcastle disease exists into the United States

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Executive Summary

For any country that wants to export table eggs into the United States from regions where Exotic Newcastle disease (END) is considered to exist, the current 9 CFR 94.6 regulation requires (1) that sentinel birds must be present in the flock of origin for at least 60 days before the export certificate is signed, (2) that there was at least one sentinel bird per 1,000 poultry, with at least 30 sentinel birds per house and (3) sentinel birds must remain free of clinical and immunological evidence of END as demonstrated by negative hemagglutination inhibition tests conducted on blood samples drawn at 10-day intervals by a salaried veterinary officer of the national government of the region of origin. Alternatively, this same regulation allows at least 10 percent of birds to be randomly swabbed for the purpose of ascertaining the absence of END in a flock.

This document submits an argument that these requirements do not provide the desired level of assurance that END virus is absent in a flock and recommends specific changes. The sentinel bird approach is not a reliable indicator of END virus infection since seroconversion could be a result of exposure to velogenic END virus as well as exposure to lentogenic field virus or vaccine virus. The alternative option of 10 percent sampling of the population is statistically inefficient, logistically burdensome and provides limited assurance about the absence of END virus in a flock. Instead, a more efficient and effective sampling methodology is offered based on targeted sampling of cull birds (defined as sick and dead birds removed from the flock for any reason) to detect END virus.

Targeted sampling of cull birds detects infection more rapidly than random sampling or sentinel birds and with greater efficiency. The relative efficiency of targeted sampling of cull birds can be considerably higher than that of random sampling of live birds. Appendix A shows that the relative efficiency of the proposed targeted sampling to the current random sampling is 125:1. In addition, targeted sampling has a higher probability of detecting END infection when infection exists in a poultry flock than that of random sampling and sentinel birds combined. It also provides more biological assurances about the absence of END virus when infection is absent than random sampling and the use of sentinel birds can offer together. Targeted sampling is based on the important biological factor of disease clustering around a few individuals (cull birds).

The rationale behind targeted sampling of cull birds is based on the biological assumption that a change in disease status of a flock will be reflected by an increase in morbidity and/or mortality of birds in the flock. That is, if infection does exist, it is most likely to exist in the population of cull birds. By focusing on cull birds, the likelihood of detecting new disease or a change in nature of an existing one becomes progressively higher as infection spreads in the flock. Appendix B shows that if infection is present in the flock at a low prevalence of one in one thousand, the prevalence of infection in the cull bird population is 100 times higher. Thus, testing the smaller population of cull birds is more efficient and effective than testing the larger population of healthy live birds at random. The proposed targeted sampling scheme for disease surveillance calls for testing one cull bird for each 10,000 birds in a poultry house.

Appendix A outlines the mathematical basis for the proposed targeted sampling scheme and Appendix B presents a formal mathematical derivation of it. A stochastic model was developed to assess the efficiency of targeted sampling versus random sampling in detecting END infection using a typical US commercial poultry farm of size of 100,000 birds. The model assumes a background weekly cull rate under normal conditions of 0.1 percent (that is one out of every 1,000 birds dies or gets sick per week in the flock for a reason other than END infection). Assuming that the flock has a low END infection prevalence also of 0.1 percent (that is, one out of every 1,000 birds dies or gets sick per week as a result of END infection), the probability of detecting infection by random sampling at least 10 percent of the 100,000 live birds (i.e., 10,000 birds) was 0.9999 (95% confidence interval: 0.999823, 1). In contrast, only 80 cull birds would be required to be tested under the proposed method of targeted sampling to achieve the same probability of detecting END at the same 95% confidence as random sampling. Hence, the efficiency is 125:1 (10,000/80=125) for targeted sampling over random sampling in detecting the same level of introduced disease.

The original language of 9CFR 94.6 is proposed for change as follows:

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(ix) And, if the eggs were laid in any region where END is considered to exist (see paragraph (a) of this section):

(A) No END occurred on the premises of origin or on adjoining premises for at least 21 days before the certificate was signed.

(B) There is no evidence that the flock of origin was exposed to END for at least 21 days before the certificate was signed.

(C) The eggs are from a flock of origin found free of END in the following way:

1. At least one cull (sick or dead) bird for each 10,000 live birds (occupying each poultry house certified for exporting table eggs) has been tested for END virus during each 7 day period beginning at least 21 days before the certificate was signed.
2. No clinical or immunological evidence of END was demonstrated by either: (a) embryonated egg inoculation technique from tissues of dead birds or (b) negative hemagglutination inhibition tests conducted on blood samples of sick birds collected by a salaried veterinary officer of the national government.
3. The tests were conducted in a laboratory approved to conduct the tests by the national government.
4. All results from procedures above were negative for END.

Additional language may include:

5. The normal cull rate of birds of every exporting poultry house within the exporting farm must not exceed one in 1000 per week at any time beginning at least 21 days prior to any shipments of table eggs into the United States and for as long as exportation is active.

6. All table eggs are washed and disinfected before shipping.

Exotic Newcastle Disease – A General Overview[1]

Definition

Velogenic Newcastle disease, also known as Exotic Newcastle disease, is the most severe form of Newcastle disease and is likely the most serious disease of poultry throughout the world. In chickens it is characterized by lesions in the brain or gastrointestinal tract, morbidity rates near 100 percent, and mortality rates as high as 90 percent in susceptible chickens. Neurologic signs or severe depression are the most obvious clinical sign, and some nonvaccinated birds may be found dead with no detected sign of prior illness.

Etiology

Newcastle disease viruses occur as three pathotypes: lentogenic, mesogenic, and velogenic, reflecting increasing levels of virulence. The most virulent (velogenic) isolates are further subdivided into neurotropic and viscerotropic types. The velogenic isolates (the subject of the analysis presented here) are considered exotic to the United States.

The Newcastle disease viruses belong to the Paramyxoviridae virus family and, like other members of this group, possess two surface proteins that are important to the identification and behavior of the virus. The first, hemagglutinin/neuraminidase (HN) is important in the attachment and release of the virus from the host cells in addition to its serologic identification. The other very important surface protein is the fusion (F) protein, which has a critical role in the pathogenesis of the disease. There are at least nine known types of avian paramyxoviruses based on the antigenic makeup of the hemagglutinin. Newcastle disease virus is the prototype virus for Type 1 avian paramyxoviruses.

Host Range

Inapparently infected carriers that are the most likely source for introduction of END include numerous species of exotic pet and exposition birds, waterfowl, and domestic poultry [8]. A persistent carrier state has been demonstrated in psittacine (8) and in certain other wild birds [9] whereas virus can be recovered from chickens for shorter periods of time, usually 14 days or less.

Although people may become infected with END virus, the resulting disease is typically limited to conjunctivitis.

Geographic Distribution

END is endemic in many countries of Asia, the Middle East, Africa, and Central and South America. Some European countries are considered free of END. END has caused high mortality in wild cormorants in Canada and the United States.

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[1] What is presented in this section has been taken primarily (and often verbatim) from the following two sources (1) Velogenic Newcastle Disease. Foreign Animal Diseases, Committee on Foreign Animal Diseases of the United States Animal Health Association. Richmond, VA. 1998. pp 396-405, and (2) Velogenic Newcastle (Exotic Newcastle disease, Asiatic Newcastle disease) http://www.vet.uga.edu/vpp/gray_book/FAD/VND.htm.

Transmission

In many parts of the tropics END is recurrent in the poultry populations. One possible cause of that recurrence is that birds are infected from a wild bird reservoir. Additional studies will be required before it can be established which species, if any, are true carriers and which are only transiently infected. It is not known whether the occurrence of END in wild birds moving in international trade can be reduced by avoiding the capture of certain species or their collection at certain time periods or places.

Once introduced into poultry, the virus spreads farm-to-farm by the movement of inapparently infected poultry species. END is spread primarily through direct contact between healthy birds and the bodily discharges of infected birds. The disease is transmitted through infected birds' droppings and secretions from the nose, mouth, and eyes. END spreads rapidly among birds kept in confinement, such as commercially raised chickens. END virus can also be spread by contaminated objects such as boots, sacks, egg trays, and crates; or by flies [1] or mice.

High concentrations of the END virus are in birds' bodily discharges. Therefore, the disease can be spread easily by mechanical means. Virus-bearing material can be picked up on shoes and clothing and carried from an infected flock to a healthy one. The disease is often spread by vaccination and debeaking crews, manure haulers, rendering truck drivers, feed delivery personnel, poultry buyers, egg service people, and poultry farm owners and employees. The END virus can survive for several weeks in a warm and humid environment on birds' feathers, manure, and other materials. It can survive indefinitely in frozen material. However, the virus is destroyed rapidly by dehydration and by the ultraviolet rays in sunlight. Reports from England [5] claim that the virus can be wind-borne under certain conditions.

Although people may become infected with END virus, the resulting disease is typically limited to conjunctivitis. Recovery is usually rapid, and the virus is no longer present in eye fluids after four to seven days. Infections have occurred mostly in laboratory workers and vaccinating crews with rare cases in poultry handlers. No instance of transmission to humans through handling of or consuming poultry products is known. Individuals with conjunctivitis from END virus should not enter poultry premises or come in contact with live avian species.

Incubation Period

The incubation period for Newcastle disease after natural exposure varies from two to 15 days. For END in chickens, an incubation period of two to six days is common. The incubation period in other species of birds may be longer.

Clinical Signs

END is a devastating malady in unvaccinated chickens of any age. The virus affects primarily the respiratory, nervous, and digestive systems. The first sign in laying chickens is usually a marked decline in egg production followed within 24 to 43 hours by high death losses. At the onset, 10-15 percent of a flock may be lost in 24 hours. After seven to 10 days, deaths usually subside, and birds surviving 12 to 14 days generally do not die but may

display permanent paralysis and other neurologic signs. The reproductive system may be permanently impaired, and thus egg production does not return to previous levels. In vaccinated chickens, or chicks protected by parental antibodies, the clinical signs are less severe and are proportional to the level of protective antibodies.

With viscerotropic strains, edema of the head, especially around the eyes may become apparent after birds have been sick for two or three days [4]. This edema usually does not involve the comb and wattle to the extent of highly pathogenic avian influenza (HPAI). A dark ring sometimes forms around the eye, probably due to cyanosis and poor blood circulation in the edematous tissue. This "black eye" appearance is especially visible in white chickens.

Bile-stained, greenish-dark diarrhea may be noted two to three days after onset of illness. Some birds in an affected flock usually have diarrhea throughout the course of the disease. The most noteworthy clinical sign in unvaccinated flocks is sudden death without prior indications of illness. The peracute onset often causes the owner to suspect poisoning.

Respiratory distress and signs of neurological disturbances, such as drooping wings, torticollis, and ataxia may not be as marked as they are with the neurotropic forms of the disease. However, these neurologic signs are frequently observed in chickens that survive infection with the viscerotopic strains for two or three weeks. Because of lack of experience with viscerotropic strains, poultry owners throughout the United States and Canada may not consider Newcastle disease as a possible diagnosis unless they see the neurologic signs they have seen with the domestic neurotropic viruses.

Neurotropic strains cause respiratory signs soon followed by neurologic signs, including muscular tremors, paralysis of legs or wings, torticollis, and opisthotonos. There is a marked decline in egg production but ususally no diarrhea. Disease signs may differ markedly, depending on the host species. Psittacines or pigeons infected with the viscerotropic strains of virus may display neurologic signs typical of the disease caused by the strains of neurotropic ND in chickens [3]. These same viscerotropic viruses may cause typical signs and lesions of VVND when inoculated into chickens [2]. In some species, such as finches and canaries, clinical disease may not be observed.

Gross Lesions

No gross lesion may be observed in many of the first birds dying in a commercial poultry operation. Peracute deaths are generally due to collapse or dysfunction of the reticuloendothelial system before discernible gross lesions have developed. There is no pathognomonic gross lesion for END, but, generally, sufficient lesions can be found to make a tentative diagnosis if enough birds are examined [6]. Because of the marked similarities between the gross lesions of END and HPAI, a final diagnosis in the first flocks to be sampled must await virus isolation and identification. In a continuing outbreak where numerous flocks are involved, gross observations may eventually be all that is necessary when typical lesions are present.

Edema of the interstitial tissue of the neck, especially near the thoracic inlet, may be

marked. After the trachea and esophagus are exposed during necropsy examination, straw colored fluid may drip from these tissues. Congestion and occasionally hemorrhage may be seen in the trachea generally corresponding to the rings of cartilage.

Proventriculus Petechial and small ecchymotic hemorrhages may be present on the mucosa of the proventriculus. These small hemorrhagic foci tend to be found near the base of the papillae and concentrated around the posterior and anterior orifices.

Intestine Peyer's patches, cecal tonsils, and other focal aggregations of lymphoid tissue in the gut wall usually are markedly involved and are responsible for the term viscerotropic applied to this form of Newcastle disease. These areas progressively become edematous, hemorrhagic, necrotic, and ulcerative. In chickens that have died from END, these involved lymphoid areas can often be observed without opening the gut.

Reproductive System

Ovaries may be edematous, hemorrhagic, or degenerated. Yolk peritonitis can frequently be observed in layers as a result of END, and rough, misshapen eggs are frequently laid by recovering hens.

Neurotropic strains of END may cause few gross lesions other than in the trachea and lungs. There will be no gross lesion in the brain of diseased birds. Gross lesion patterns usually differ markedly between the disease caused by the viscerotropic and neurotropic velogenic viruses.

Morbidity and Mortality

Clinical END is most severe in chickens, peafowl, guineas, pheasant, quail and pigeons. Turkeys may develop a milder form of the disease. Severity of disease in psittacine and passerine birds is variable. In susceptible chickens, the morbidity and mortality rates can be as high as 100 percent and 90 percent, respectively. In some species such as finches and canaries, clinical disease may not be observed.

Diagnosis

Field Diagnosis

A tentative diagnosis of END may be made on the basis of history, clinical signs, and gross lesions, but because of similarities to other diseases such as fowl cholera and highly pathogenic avian influenza, confirmation requires virus isolation and identification.

Specimens for Laboratory

Virus can readily be recovered from sick or recently dead birds. Swabs are the most convenient way to transfer END virus from tissues or secretions of the suspect bird to brain and heart infusion broth or other cell culture maintenance medium containing high levels of antibiotics. Trachea, lung, spleen, cloaca, and brain should be sampled. Swabs should be inserted deeply to ensure obtaining ample epithelial tissue. If large numbers of dead or live birds are to be sampled, cloacal swabs from up to five birds can be pooled in the same tube of broth. An alternate technique is to place 0.5 cm^3 of each tissue into the broth. If the

specimens can be delivered to a laboratory within 24 hours, they should be placed on ice. If delivery will take longer, quick-freeze the specimens and do not allow them to thaw during transit.

Laboratory Diagnosis

In the laboratory, virus isolation is attempted by inoculating nine- to 11-day-old embryonating chicken eggs. Chorioallantoic fluid (CAF) is collected from all embryos dying after 24 hours postinoculation and tested for hemagglutination (HA) activity. If positive, the hemagglutination-inhibition (Hl) test is used with known NDV-positive serum to confirm the presence of NDV in the CAF (3). If NDV is found, it is characterized by inoculating 4- four- to six-week-old chickens free of ND antibodies with the suspect CAF by swabbing the cloaca, instilling into the nares or conjuctival sac, or injecting into the thoracic air sac. If END virus is present, the inoculated chicks usually die in three to seven days, revealing typical visceral lesions on postmortem examination. Neurotroph ic VVD viruses will cause severe neurologic and respiratory signs in inoculated chickens but no visceral lesions. If no bird dies in 10 days, the NDV is not considered to be the velogenic, viscerotropic type but is either a lentogen or mesogen.

Differential Diagnosis

END in poultry can be confused with HPAI, infectious laryngotracheitis, fowl cholera, coryza and poisoning.

Vaccination

Vaccination with viable or inactivated oil emulsion vaccines, or both, can markedly reduce the losses from END in poultry flocks. If eradication of the virus is not the goal of the control program, vaccines can be used to lessen the impact of the disease. Their use, however, can make the complete eradication of the virus much more problematic by increasing the difficulty of identifying infected flocks. There is little doubt, however, that vaccination makes the flock more refractive to infection when exposed and reduces the quantity of virus shed by infected flocks.

Control and Eradication

Before 1972, END was introduced into the United States on several occasions by unrestricted introduction of exotic pet birds, especially psittacine birds. Because pet birds are not usually associated with domestic poultry, END outbreaks were rare. Since 1973, restrictions on the importation of exotic birds requiring the quarantining and testing of imported birds in approved quarantine facilities have reduced but not eliminated the threat of END in the United States. Illegally imported exotic bird species remain the source of frequent outbreaks of END in private or commercial aviaries.

The establishment of a strict quarantine and destruction of all infected and exposed birds with financial indemnification for losses followed by thorough cleaning and disinfection of premises were the main features necessary for eradication of END virus from the poultry producing area of southern California. Flocks may be safely and humanely destroyed using carbon dioxide in air-tight chambers and the carcasses disposed of by burying, composting, or rendering, depending upon the geographic area and the numbers involved. The END

virus has been recovered from effluent water for as long as 21 days and from carcasses for seven days when the daytime temperatures were over 90 F. It is recommended that premises be kept free of domestic poultry for an additional 30 days after cleaning and disinfection are completed.

Insects and mice associated with the poultry should be destroyed before depopulation of a flock begins. Usually 48 hours is sufficient to control these vectors. As soon as all birds are killed and the manure and feed removed, all equipment and structural surfaces should be thoroughly cleaned using high-pressure spray equipment. The entire premises should then be sprayed with an approved residual disinfectant such as the cresylics or phenolics. Preliminary disinfection will probably inactivate most of the viruses on the surface of floors, equipment, cages, etc., but no disinfectant is effective unless it is applied to scrupulously cleaned surfaces free of all organic material.

Cleaning and disinfecting commercial poultry premises are time-consuming and expensive operations. All manure must be removed down to a bare concrete floor. If the floor is earthen, at least the top one inch of soil should be removed with the manure. Manure can be safely disposed of by burying it at least five feet deep or by composting. If composting is used, the manure piles should be tightly covered with black polyethylene sheets in a manner to prevent access by birds, insects, scavengers and rodents during composting, these piles of manure should remain tightly covered and undisturbed at least 90 days during warm weather and for longer period during cold weather. Recent studies indicate that proper composting can decompose carcasses and manure, and thus inactivate viruses in only a few weeks.

Feathers, usually numerous around commercial poultry premises, can be burned outside the buildings, and in some cases inside, with the careful use of a flame thrower, or they can be removed and the area wet down with disinfectant. The hot sun and high daytime temperatures will assist in destroying the virus in the area of the houses. Extremely cold temperatures will make the cleaning and decontamination process much more difficult, and the results more uncertain.

In 1997, because neither the neurotropic or viscertropic strain of END was known to exist in the United States, USDA-APHIS declared both types to be exotic and therefore indistinguishable as to the response of disease control officials should they occur in the United States.

Surveillance Methods Used to Detect END Virus in Flocks

Detecting END virus in an unvaccinated flock is easier than detecting it in an infected but apparently healthy flock that has been vaccinated. Vaccination does not necessarily prevent infection, but it may lessen the impact of the disease substantially - by reducing the susceptibility to infection, severity of disease, morbidity, mortality, lesions, and the duration and quantity of virus shed, etc. These factors complicate early detection of END virus. Therefore, surveillance to detect disease must rely to a greater extent on sampling and laboratory diagnostic methods based on virus detection [10].

Several surveillance methods may be used to detect END virus in a vaccinated, infected but apparently healthy flock. We discuss three such methods as they pertain to the current 9 CFR 94.6 requirements and recommended changes. Methods discussed are: (1) sentinel birds, (2) random sampling of live birds, and (3) targeted sampling of cull birds.

Sentinel Birds

The sentinel bird method of virus detection is required in the current 9 CFR 94.6 regulation. Sentinel birds are unvaccinated and pathogen-free birds which are placed in vaccinated flocks to serve as a virus detection system for END [10]. Specifically, the current 9 CFR 94.6 regulation requires (1) that sentinel birds be present in the flock of origin for at least 60 days before the export certificate is signed, (2) that there was at least one sentinel bird per 1,000 poultry, with at least 30 sentinel birds per house, and (3) that the sentinel birds remained free of clinical and immunological evidence of END as demonstrated by negative hemagglutination inhibition tests conducted on blood samples drawn at 10-day intervals by a salaried veterinary officer of the national government of the region of origin.

The rationale behind the placement of sentinel birds in a possibly infected flock is that, in *most* instances, if velogenic form of END virus is present, sentinel birds typically will be highly susceptible to the virus and will easily become infected and die within a week or so after placement. However, if the other forms (lentogenic, mesogenic) of END virus are present, sentinel birds may not be a reliable method of detection of END virus for the following reasons:[2]

1. In some cases it is difficult to place sentinel birds so they are adequately exposed to any END virus that may be in the flock, especially in caged-layer flocks and when small numbers of birds are infected within very large flocks of poultry.
2. Seroconversion may not be a result of exposure to velogenic form of END virus but could be a result of exposure to lentogenic field virus or live vaccine.

In addition, it is sometimes difficult to obtain unvaccinated and pathogen-free birds to serve as sentinel birds in a vaccinated flock.
Thus, sentinel birds do not provide the desired level of assurance of the absence of virus in a flock, particularly when the virus is not virulent.

[2] Reference: Based on discussions with an END panel of experts (list names of experts and their affiliation, if known) assembled to deal with END-related issues during the 2003 END outbreak in California.

Random Sampling

Random sampling and swabbing of live birds involves choosing a sample of *n* birds to test at random from the flock of susceptible birds. If all *n* tests turn out to be negative, the virus is considered (with some pre-set statistical confidence) to be absent in the flock. While this approach is statistically valid, it can be grossly inefficient since it often requires large sample sizes to attain the desired level of statistical confidence. This is particularly true when the prevalence of infection in the flock is low.

Even with large sample sizes, random sampling provides little assurance of the absence of infection when testing finds no infection in the sampled population. The primary reason for random sampling's inefficiency in detecting infection in a flock when it is present is that it assumes that infection is uniformly distributed in the population. However, under field conditions infection is typically clustered around a few individuals in a population, i.e. most populations comprise varying proportions of healthy, subclinically diseased, and clinically diseased individuals, with the proportions being subject to change over time [12]. Violation of the uniformity of infection assumption under random sampling results in incorrect sample size determinations, inflated statistical confidence and ignoring important biological and epidemiological considerations.

Random sampling is utilized primarily to ensure desired statistical properties of estimators, e.g., unbiasedness and minimum variance, when estimating population parameters such as disease prevalence. However, if the objective is to detect disease or to demonstrate freedom from it, such statistical properties are not important or even relevant. The only interest in random sampling is to ensure adequate sampling coverage of the population. Thus, for the purpose of finding disease in a flock, random sampling is not necessary. In fact, it is one of the most inefficient methodologies for finding disease and is primarily utilized as a baseline with which to compare relative efficiencies of other sampling methodologies.

The regulation in 9CFR 94.6 alternatively allows a country to randomly swab at least 10 percent of birds for the purpose of ascertaining the absence of END in flocks that will be the source of table eggs for export. The basis for this requirement, presumably, is to ensure that a large enough unbiased sample of birds from the population will be selected for swabbing to find END virus if it is present. However, this requirement is statistically inefficient, logistically burdensome and provides limited assurance about the actual absence of END infection in a flock. This position is based upon the following:

1. Sampling is required to be conducted, presumably once, within the last 60 days prior to shipment, yet flocks could become infected after sampling and prior to egg shipment, and thereby providing limited assurance that END virus is not present in the eggs[3];
2. Current 9 CFR 94.6 regulations specifically require that at least 10 percent of the flocks must be sampled, regardless of flock size, and thus proportionally links the size of the sample to the size of the population, which is theoretically unfounded.

More importantly, however, the current requirement of random swabbing of birds does not

[3] Reference: Based on discussions with an END panel of experts assembled to deal with END-related issues during the 2003 END outbreak in California.

provide a reasonable biological and epidemiological level of confidence about the absence of END infection over time even if more than 10 percent of the population is swabbed. As mentioned previously, disease is not uniformly distributed in a flock but is typically clustered around a few individuals.

Ascertaining absence of infection in the flock over time requires continuous, or at least periodic, sampling. Should random sampling be used systematically over time, its inefficiency is further exaggerated. Sampling may well exceed 10 percent of the population, particularly when the prevalence of the disease is low in a vaccinated flock, where there is little virus shedding and when no clinical signs are displayed. Under certain limited circumstances, random sampling may provide the desired level of assurance of the absence of infection in a flock but only after increasing the sample size to unreasonably high levels.

Targeted Sampling

A more efficient approach to detect END virus in a large vaccinated flock is through targeted sampling and monitoring of cull birds. Cull birds are defined in this monograph as birds removed from the flock for death or sickness for any reason[4]. The biological basis for targeted sampling is that if infection does exist in the flock, it is most likely to exist in the population of sick and dying birds. In the example of Appendix B, a prevalence of 0.1 percent in the flock results in a prevalence of 100 times higher in the population of dead birds from that flock. Thus, the assumption underlying targeted sampling is that a change in disease status of a flock will be reflected by an increase in morbidity and/or mortality of birds in the flock. By focusing on sick and dying birds, i.e. cull birds, the likelihood of detecting new disease or a change in nature of an existing one becomes progressively higher as infection spreads in the flock. Another fundamental condition for targeted sampling is that disease status in the flock under normal conditions must be known in order to recognize significant changes and irregularities in the health status of the flock.

Targeted sampling of cull birds detects infection quicker than random sampling or sentinel birds and with a greater efficiency. Depending on the situation, the relative efficiency of targeted sampling of cull birds can be considerably higher than that of random sampling of live birds. In the example of Appendix A, the relative efficiency of the proposed targeted sampling to the current random sampling is 125:1. In addition, targeted sampling has a higher probability of detecting END infection when infection exists in a poultry flock than that of random sampling and sentinel birds combined. Appendix B shows that the probability of detecting infection in the dead bird population is 0.3 as opposed to 0.003 in the healthy live bird population. It also provides more biological assurances about the absence of END virus when infection is absent than random sampling and the use of sentinel

[4] Disease biology is an important consideration with END virus. This is a highly contagious disease where serological sampling may be confounded by lentogenic strains of the virus or by vaccinations while clinically normal but infected birds may shed virus only intermittently. If the choice of testing is to look for the presence of virus in clinically normal flocks, the prevalence of birds shedding at any given time may be in fractions of a percent while the number of birds required to sample to detect the virus may be very large. In contrast, the presence of END virus is likely to be quite high in the population of sick or dead birds if the flock is truly infected with this virus. The number of birds needed to sample is quite low. Thus, random sampling would require a prohibitively large sample size with very poor statistical confidence of finding the virus whereas sampling the targeted population of sick and dead birds would require a much smaller sample size and provide a much higher confidence of identifying END virus.

birds can offer together. This is because targeted sampling does not assume that disease is uniformly distributed in the population. Rather, it is based on the important biological factor of disease clustering around a few individuals (cull birds). It is this cluster of birds that is the focus of targeted sampling.

Proposed Changes to 9CFR 94.6 Sampling Methodology – The "5-5 by 100,000 Rule"

Rationale

A targeted sampling method, dubbed the "5-5 by 100,000 Rule", is proposed to replace the current 9CFR 94.6 regulation which uses random sampling methodology to demonstrate freedom from disease. This alternative sampling method involves collecting five dead and five sick birds each week from each poultry house (of 100,000 birds)[5] (therefore its name the "5-5 by 100,000 Rule") in a large poultry farm to rule out the presence of END infection.

For houses with fewer or more birds, the rate of testing under the proposed rule is to test one cull bird per 10,000 live birds. For example, if a poultry house has 50,000 birds, then at least five cull birds (three dead and two sick or two dead and three sick) should be tested; if the size of the poultry house is 160,000 then 16 cull birds (eight dead and eight sick) should be tested, and so on.

This sampling methodology is patterned after what is currently being done by commercial poultry operations in the United States to monitor overall disease makeup of a flock. The US system presumably cannot be made into a formal requirement in the 9CFR 94.6 regulations because verification of such a system may entail examination of proprietary data. The proposed "5-5 by 100,000 Rule" (and its associated rate of testing one cull bird for every 10,000 live birds) is the closest working image of that system. Virtually all modern poultry farms in the U.S. use a method whereby advanced statistical control charts for a variety of parameters are utilized for monitoring overall flock health. Examination of cull birds is a specific parameter utilized for detecting the introduction of a new disease to a flock or changes in incidence of existing diseases. Cull birds are tested only when the "process goes out of statistical control". This point is reached when the cull rate goes outside the set upper limits, which is usually one and a half to two standard deviations from the mean.

In the absence of END, typical commercial poultry farms in the U.S. experience a 0.1 per cent weekly cull rate (one out of every 1,000 birds dies or gets sick for any reason). Since most, if not all, well-maintained commercial poultry operations in the U.S. are collecting these cull birds on a daily basis as an integral part of their comprehensive flock health programs; the proposed change to targeted sampling strategy does not require additional

[5] A house may be defined as a single structure housing a flock of birds, usually of the same age, that may or may not be connected by way of a corridor to other structures housing birds. If there are multiple structures connected by a corridor, to facilitate servicing or for other reasons, then each of these individual structures should be considered a separate house and should be monitored separately for the purposes of recording mortality.

effort beyond what is standard operating procedure in the poultry industry. A slight difference in implementation does exist, however. The proposed method calls for weekly testing of cull birds whereas testing of cull birds in large U.S. poultry operations is conducted only when needed (when the monitored health indicator goes out of statistical control).

Mathematical Basis

The mathematical basis for the proposed change in 9CFR 94.6 sampling methodology from random sampling to targeted sampling is outlined in Appendix A. A formal derivation of the general underlying methodology of targeted sampling of cull birds is given in Appendix B. Appendix A also assesses the confidence[6] associated with the "5-5 by 100,000 Rule" in detecting END using a quantitative stochastic simulation model. Appendix B compares the relative efficiency of the proposed (targeted sampling) and the existing methods (random sampling) in finding END infection in a large vaccinated layer flock of approximately 100,000 birds.

Assuming that a typical commercial poultry farm in the U.S. with a background 0.1 per cent weekly cull rate under normal conditions (one out of every 1,000 birds dies or gets sick per week for any reason other than END infection) has END infection introduced to the flock at the rate of 0.1 percent (one out of every 1,000 birds is infected), Appendix A shows that the probability of detecting infection with random sampling of at least 10 percent (10,000) of every 100,000 live birds in a 60-day period as required by the current 9CFR94.6 regulation is 0.99998, with a 95% confidence interval of (0.999823, 1). For the same 60-day period, only 80 cull birds would be required to be tested under the proposed method of targeted sampling. The probability of detection under the proposed targeted sampling of cull birds is 0.99999, with 95% confidence interval of (0.99999, 1).

These two probabilities are the same up to the 5^{th} decimal point (with the latter slightly higher than the former), but the number of birds required by each method is vastly different. Over 60 days (the frequency of sampling under the current regulation) only 80 cull birds would be required under the proposed rule to detect infection as opposed to 10,000 live birds under the current regulation. Hence, the efficiency is 125:1 (10,000/80=125) for targeted sampling over random sampling in detecting the same level of introduced disease.

Proposed Modification of 9CFR 94.6 Language

The original language of 9CFR 94.6 is proposed for change as follows:

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[6] Confidence may be interpreted slightly differently for the two different infection situations: (1) When infection is assumed to exist in the flock, confidence is typically interpreted as the probability of detecting infection *and* the confidence associated with that probability; and (2) When infection is absent, confidence may be interpreted as a measure of "assurance" about the absence of infection. In case (2), confidence is synonymous with assurance. However, when the status of the presence or absence of infection is not known, confidence is interpreted as the totality of the likelihood of finding infection when it exists *and* the level of assurance when it doesn't.

(ix) And, if the eggs were laid in any region where END is considered to exist (see paragraph (a) of this section):

(A) No END occurred on the premises of origin or on adjoining premises for at least 21 days before the certificate was signed.

(B) There is no evidence that the flock of origin was exposed to END for at least 21 days before the certificate was signed.

(C) The eggs are from a flock of origin found free of END in the following way:

7. At least one cull (sick or dead) bird for each 10,000 live birds (occupying each poultry house certified for exporting table eggs) has been tested for END virus during each 7 day period beginning at least 21 days before the certificate was signed.
8. No clinical or immunological evidence of END was demonstrated by either: (a) embryonated egg inoculation technique from tissues of dead birds or (b) negative hemagglutination inhibition tests conducted on blood samples of sick birds collected by a salaried veterinary officer of the national government.
9. The tests were conducted in a laboratory approved to conduct the tests by the national government.
10. All results from procedures above were negative for END.

Additional language may include:

11. The cull rate of birds of every exporting poultry house within the exporting farm must not exceed one in 1000 per week at any time beginning at least 21 days prior to any shipments of table eggs into the United States and for as long as exportation is active.

All table eggs are washed and disinfected before shipping[7].

It is important to re-emphasize to sample every poultry house that is approved for export of table eggs. The primary reason for testing every house on a continuous basis is to detect intermittent shedding of virus or recent introductions of END virus. Birds which have died from obvious management factors or other non-infectious causes (e.g. fractures, predation) should be excluded from sampling. In the absence of mortality, sick birds should be selected for sampling.

Summary

[7] The panel of experts consulted on this issue suggested that washing and disinfection of eggs be included as a requirement in the revised regulation rather than being supplemental. The panel also believed that washing and disinfection of eggs is common practice in U.S. table egg industry.

If END infection is present at a low prevalence in a flock, random sampling is an inefficient method for detecting END infection in large poultry flocks. It requires unreasonably large numbers of birds to be sampled in order to attain an acceptable level of statistical confidence to detect infection. Furthermore, when END infection is not present in a flock, random sampling does not provide the desired level of biological assurance of its absence even if a large number of birds test negative to the virus.

In contrast, more efficient and effective methods of monitoring flocks for potential disease incursions and other irregularities involves the use of basic health indicators such as morbidity, mortality, egg production, and food and water intake in individual birds. Examination of cull birds is one such method employed to detect the introduction of a new disease to a flock or changes in incidence of an existing one. The rationale behind targeted sampling of cull birds is based on the fundamental biological assumption that a change in disease status of a flock will be reflected by an increase in morbidity and/or mortality of birds in the flock. By focusing on sick and dying birds, the likelihood of detecting new disease or a change in nature of an existing one becomes progressively higher in the flock. Thus, testing the smaller population of cull birds is more efficient and effective than testing the larger population of healthy live birds. It is also logistically easier.

However, since this system presumably cannot be made into a formal requirement in the 9CFR 94.6 regulations because verification of such a system may entail examination of proprietary data, the proposed "5-5 by 100,000 Rule" (and its associated rate of testing one cull bird for every 10,000 live birds) is the closest working image of that system. This sampling method can be easily incorporated into the 9CFR 94.6 regulation and represents a significant improvement over the existing regulation outlined in the current 9CFR 94.6 to rule out the presence of END infection in a large flock. For comparable confidence in detecting infection, the efficiency of the "5-5 by 100,000 Rule" was shown to be 125 times (i.e., 12,500 percent) better than that of the random sampling of birds under the current 9CFR 94.6 regulation.

The efficiency and effectiveness of targeted sampling of cull birds depend on the probabilities of mortality and morbidity as a result of infection. Vaccine protection may decrease these two probabilities and lessen the numbers of dead and sick birds in a flock[8]. Although targeted sampling of cull birds is always more efficient than random sampling of live birds, it may not provide the desired level of assurance of freedom from disease. Additional mitigation, e.g., washing and disinfecting eggs, may further ensure that END infection, should it be in the flock but not identified by testing procedures, will not be transmitted via table eggs.

In multi-house poultry farms, flocks are unlikely to be infected in all houses simultaneously. Consequently, in the early stages of an outbreak of disease there may be no apparent increase in overall mortality for the entire farm. Therefore, careful monitoring of mortality for each house, including laboratory-based surveillance, is necessary to quickly identify potentially infected flocks and thus prevent spread of disease. For the strategy to be used as an effective disease monitoring and detection system in all poultry flocks irrespective of

[8] In non-vaccinated flocks, the rate of death due to infection may exceed 90% but may be well less than 2% in a vaccinated flock.

whether or not they are vaccinated, the rate of cull birds in each poultry house must not exceed a certain specified upper limit, such as one cull bird per 1,000 birds per week. This is the average (acceptable) rate of cull birds under normal conditions in well-maintained large U.S. commercial poultry operations. Hence, the modified regulation may require that for a poultry farm to export its table eggs, it must certify that the cull rate of birds in each poultry house that is certified for exportation of table eggs has not exceeded the rate of one cull per 1000 birds per week at any time for the past 21 days before a certificate is signed. The regulation may further stipulate that if the cull rate exceeds one cull bird per 1,000 birds per week, exportation is halted.

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APPENDIX A

Mathematical Basis of the "5-5 by 100,000 Rule"

An efficient and effective method of monitoring flocks for potential disease incursions and other irregularities is the monitoring of basic health indicators such as mortality, morbidity, egg production, and food and water intake. This system of monitoring health indicators and testing birds as needed is the primary method used by large U.S. poultry operations to establish absence of new disease and other irregularities in their flocks. Virtually all modern poultry farms in the U.S. use a method where advanced statistical control charts are utilized for monitoring overall flock health. Cull birds (defined here as the sick *and* dead birds) are tested only when the "process goes out of statistical control". This point is reached when at least one of the monitored indicators goes outside the set upper limits, which are usually one and a half to two standard deviations from the mean.

The two-phase system of monitoring and sampling is similar to the well known statistical process control (SPC) used in engineering and assembly lines. The two steps of the system are to: (1) monitor the number of cull birds in each poultry house on a daily, or at least weekly, basis; (2) test all, or at least a sample, of the cull birds whenever the observed number of cull birds exceeds a certain specified upper limit.

Determining upper limits for the daily number of cull birds in each poultry house requires knowledge about the rate of cull birds under normal conditions in that house. There may be significant variability associated with this rate both between different poultry houses within a farm as well across different farms (that is, the cull rate under normal conditions varies both within and among poultry farms). Ideally, the cull rate under normal conditions should be determined for each poultry house within a farm to help determine upper limits for the daily (or weekly) number of cull birds for that particular house[9]. Upper limits are typically set at 1.5, 2, or 2.5 standard deviations above the mean of the monitored health indicator, e.g., the mean daily (or weekly) number of cull birds. If monitoring data are not available from each poultry house individually, an overall average value of the cull rate of the farm or similar farms may be used instead. Using a generic estimate of the cull rate may result in over testing to compensate for the uncertainty associated with the estimate. Another advantage of using data collected from each poultry house is to help identify possible infection cycles for that particular house. Cycles may be weekly, monthly, yearly, seasonally, or may be business production cycles, and may vary greatly from one house to another. For example, the phase and the frequency of these cycles may differ from house to house within a farm. Estimating the cull rate in each house using its own data help reduce the added variability associated with different cycles and result in more accurate estimates. That is, identifying such cycles using data specific to the poultry house in question, removing or adjusting for them using standard statistical time-series analysis techniques, will enhance the estimate of the true number of cull birds that die and get sick under normal conditions for reasons other than disease.

[9] If monitoring is conducted and data are collected in house *i*, then the cull rate can be easily estimated from data and upper limits can then be determined accordingly.

No matter which estimate of the cull rate one uses to determine the upper limits, once upper limits of the daily (or weekly) number of birds are determined, then if the observed number of cull birds on a given day (or week) exceeds the set upper limit, testing of all (or at least a sample) of such birds is triggered to determine the possible cause(s).

As mentioned previously, under the two-phase system of monitoring and testing used by most large poultry operations in the US, testing is conducted only when the system "goes out of statistical control", i.e., when "spikes" occur in the monitored health indicators. These spikes are typically acted upon when abnormalities exceed two standard deviations of the mean of production (or other key indicator)[10], e.g., when the daily or weekly number of dead birds exceeds its upper limit set according to the standards of the particular poultry operation. However, recognizing spikes necessitates monitoring, which is a desired event that presumably cannot be required in the 9CFR regulation. Since monitoring cannot be required to identify spikes for testing, the most viable alternative is to test cull birds uniformly and systematically instead.

The "5-5 by 100,000 Rule" attempts to mimic the two-phase system by testing cull birds uniformly over time using a comparable number of birds culled under the two-phase voluntary system employed by the U.S. poultry industry. In particular, the "5-5 by 100,000 Rule" calculates the expected number of tests resulting from spikes over a year under normal conditions in a poultry house of approximately 100,000 birds and distributes them uniformly at weekly intervals over the entire year.

The "5-5 by 100,000 Rule" is patterned after typical sampling protocols employed by commercial poultry operations in the U.S. In these large poultry operations the average number of cull birds per week is one tenth of one percent (0.1 percent or one in 1000 birds). These operations collect dead and sick birds (referred to in U.S. poultry operations as "cull birds") on daily basis. A cull rate of 0.1 percent in a poultry house of 100,000 live birds translates, on average, to 100 cull birds per week, or approximately 15 cull birds per day. In the U.S., testing is triggered when the observed number of cull birds in any given day exceeds some set upper limits.

Assuming that the number of daily cull birds, X, follows Poisson probability laws, and using one and a half standard deviations above the mean[11], the upper limit is calculated by adding 15, the mean daily number of cull birds, to one and a half times the standard deviations of the mean, which, for the Poisson is (1.5)(sqrt(15)) = 5.81 or 6. The resulting number is 21. This number represents the upper limit for the daily cull birds in this particular house of approximately 100,000 birds. Thus, if the number of cull birds in this poultry house exceeds 21, a spike has occurred and testing is triggered.

The probability that testing is triggered on any given day in this house is equal to the probability that the number of cull birds (dead *and* sick birds), X, exceeds 21. Assuming that X is Poisson distributed, this probability is calculated as follows:

[10] Different poultry operations establish upper limits according to their production managements and other criteria. Most use 2 standard deviations; some use 2.5 and others 1.5 standard deviations in setting their limits.

[11] Using 1.5 instead of the more commonly used value of two standard deviations in setting upper limits results in more testing, and hence is more conservative and less risky.

$$P(X > 21) = \sum_{x=22}^{\infty} \frac{e^{-15}(15)^x}{x!}$$
$$= 1 - P(X \leq 21)$$
$$= 1 - \sum_{x=0}^{21} \frac{e^{-15}(15)^x}{x!}$$
$$= 0.053106$$

Again, this represents the probability of testing a flock of approximately 100,000 birds under normal conditions on any given day.

The number of times testing may be triggered in a given year follows a binomial distribution[12] with n = 365 days, the number of days in a year, and probability p = 0.053106. The mean of this distribution is equal to n x p = (365)(0.053106) = 20 spikes, or equivalently, 20 days out of the year where testing under normal conditions is prompted. Assuming that when testing is triggered at least the upper limit value of 21 birds are tested, then the total number of birds that will be tested in a year is equal to 21x20 = 420 birds on average[13]. Dividing by 52, the number of weeks in a year gives 8.1 or approximately 9 birds per week. Rounding up to ten birds for convenience and splitting the testing equally between dead and sick birds, the "5-5 by 100,000 Rule" follows.

Assessing confidence of detecting infection with the "5-5 by 100,000 Rule"

An electronic spreadsheet (Microsoft® Excel 2002) was used as the platform for building the quantitative model to assess the confidence in detecting infection, should infection exist in a flock, associated with the "5-5 by 100,000 Rule". Additional software (@Risk© – Risk Analysis Add-in for Microsoft Excel, Version 4.5.2 – Professional Edition) was used to provide stochasticity (i.e., probabilistic simulation components) to the model.

The stochastic model is based on the following assumptions:

- END infection exists in a large poultry house of approximately 100,000 vaccinated layers.
- To be more conservative with estimating confidence, i.e., to not over estimate confidence, a very small prevalence of infection of 0.001 (i.e., one infected bird out of every 1,000 birds) is assumed.
- At any given time *t*, an infected bird would be in one of three possible states: (1) infected but healthy-looking, (2) infected and sick, or (3) infected and dead.
- The "5-5 by 100,000 Rule" calls for sampling only dead and sick birds, i.e., no sampling of healthy birds.
- Since confidence in finding infection in healthy birds is practically zero, the model assesses confidence associated with the targeted sampling of dead and sick birds only.

[12] The binomial distribution is the most appropriate distribution to model the number of spikes (i.e., test days) in a given year.
[13] If a few more than 21 dead and cull/sick birds are tested when a spike occurs, e.g. 26, the total number of tests per year will be higher than 420.

Model parameter values

It is to be noted again that in the U.S. large poultry operations collect and remove dead and sick birds from their flock on a daily basis. These birds are called "cull birds". When cull birds are tested in the U.S. poultry operations, no distinction is made between sick and dead birds since sick birds are killed upon collection and placed together with other dead birds. In other countries, poultry operations may distinguish between dead and sick birds at the time of testing. Since confidence associated with each situation may be different, the model assesses confidence of detecting infection for each situation separately. It turns out, however, that the difference in the model output is minimal and the output presented at the end of this monograph is that of no distinction is made between dead and sick birds at the time of testing, which reflects the testing conducted in large U.S. poultry farms.

Poultry experts[14] provided estimates for the conditional probabilities for each of the three states used in the model: infected but healthy-looking; infected and sick; and infected and dead. A fourth possible state of disease, "recovered from infection", was not considered in this model. The size of the flock used in this model was 100,000 birds[15]. The model assumed that at the time of sampling, the conditional probability that a bird was infected but healthy-looking (i.e. a bird in incubation phase of END) was 0.005 (five out of every 1,000 infected birds). The conditional probability that an infected bird will be sick (bird in clinical illness phase of disease) was 0.60 (60 percent of clinically ill birds will be alive). The conditional probability that an infected bird will be dead (bird in clinical illness phase of disease) is 0.395 (39.5 percent of clinically ill birds will be dead; default probability of 1.0-0.005-0.60). Two other conditional probabilities were needed to complete the model. The probability a bird dies under normal conditions, i.e., bird dies of a condition other than END infection, is approximately 0.001 (one bird out of every 1,000 birds). Similarly, the model assumes that the probability that a bird gets sick for reasons other than END infection is also 0.001. With these assumed conditional probability values, the probability of detecting END infection can be easily calculated along with its associated confidence, i.e., the totality of the likelihood of finding infection when it exists *and* the level of assurance when it doesn't, under various targeted sampling schemes.

Confidence associated with the "5-5 by 100,000 Rule"

Figure 1 shows the cumulative distribution of the confidence[16] associated with the "5-5 by 100,000 Rule" in detecting END infection in a large flock of 100,000 vaccinated layers assuming a set prevalence of infection of 0.001 (one infected bird out of every 1,000 birds). The mean probability of detection of this distribution is 96.47% (0.9647) associated with 81% (0.81) confidence. In other words, on average, there is a greater than 80% confidence that in more than 96 out of every 100 times that this quantity of birds is sampled on a weekly basis for the presence of END that, if the disease is present in the flock at the time of sampling, that it will be detected. The 95th percentile is 0.9999986. That is to say, with

[14] Poultry Experts consulted are: Dr. Max Brugh, D.V.M., Ph.D., Poultry Health Specialist (A Private Consultant); Dr. Lindsey Garber, D.V.M., USDA:APHIS:VS:CEAH; Dr. Eric Gingerich, D.V.M., Ph.D., (President AVEP), Penn State University.

[15] The size of 100,000 is only an example. Other sizes could have been easily used, which would result in different sampling requirements, e.g., a "3-3 Rule", "7-7 Rule", or other "x-x Rule". It is important to point out that the rate of testing under the proposed method is one cull bird for every 10,000 live birds.

[16] Confidence here should be interpreted as the probability of detecting infection and its associated confidence when infection actually exists. Figure 1 represents the ability to find infection when it exists. As such, in a sense, Figure 1 represents a lower limit on the total level of assurance or certainty that infection will not be introduced through the importation of eggs from the poultry house in question.

95% confidence the probability of detecting infection is no less than 0.9999986. Also the mode of this distribution, i.e., the most frequent value of the probability of detection, is 1.0 (100 percent). In contrast, using the same prevalence of disease but a change to a 10 percent random sampling scenario as required by current 9CFR94.6 regulation, the mean probability of detection is 0.999955 with a 95% confidence interval of (0.999823, 1). The level of confidence is comparable for the proposed and the existing methods, but the number of birds required by each method is significantly different. Only 10 cull birds per week or a total of 80 cull birds over 60 days (the frequency of sampling under the current regulation) would be required under the proposed rule, as contrasted with 10,000 birds under the current regulation.

In summary, if sampling efforts focus on cull birds and away from sampling healthy birds randomly, there is a very high probability of detecting END virus even at extremely low levels of infection among birds in a poultry house.

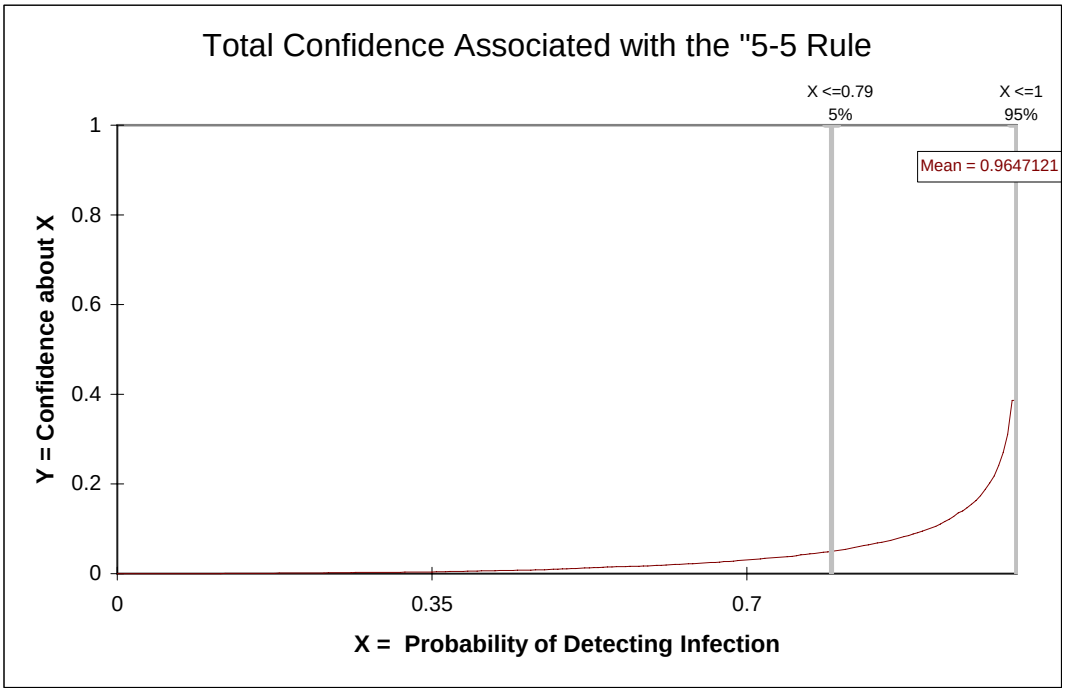

Figure 1. Cumulative distribution of the confidence associated with the “5-5 Rule” in detecting END infection in a large flock of 100,000 vaccinated layers assuming a prevalence of infection of one infected bird out of every 1,000 birds in a poultry house.

APPENDIX B

Mathematical Derivation of the Efficiency of Targeted Sampling[17]

Relative efficiencies may be determined differently for different comparison criteria. Here, the relative efficiency of the proposed targeted sampling of cull birds to the current random sampling of live birds methodology outlined in the 9 CFR 94.6 regulations will be determined based on comparison of their associated sample sizes. Specifically, the relative efficiency of the two methods is determined by dividing the minimum sample size that would be required to detect disease in the flock from using random sampling of live birds by the minimum sample size that would be required to detect disease in the flock from using targeted sampling of cull birds at the desired level of statistical confidence. That is,

$$\text{Relative\_Efficiency} = \frac{n_{\text{random}}}{n_{\text{targeted}}}, \qquad (1)$$

where n_{random} and $n_{targeted}$ represent the minimum sample sizes required to detect disease with the current random sampling methodology of live birds and the proposed targeted sampling of cull birds, respectively. The sample size criterion is highly correlated with cost and time, and is a good criterion for using in making assessments of relative efficiencies.

In order to determine the relative efficiency of the two methods in Equation (1), it is necessary to determine n_{random} and $n_{targeted}$. This, in turn, requires estimates of the prevalence of infection for each of the two populations of live birds and cull birds. It is necessary to develop some notation for the development of the theoretical steps for the estimation of prevalence and sample sizes to estimate the relative efficiency of the two sampling methods.

Appendix B model notation

Variables for the development of the theoretical steps in the model that will measure the relative efficiency of the two sampling methods are defined as follows:

X_{NC_i}: Weekly number of cull birds in house i, where $i = 1, 2, \cdots, 10,$ under normal conditions.

Y_{DC_i}: Weekly number of dead birds in house i, where $i = 1, 2, \cdots, 10,$ under disease conditions.

$Z_{Disease\text{-}\ dead_i}$: Weekly number of cull birds in house *i* that have been removed from flock solely because of disease under disease conditions.

p_{NC_i}: The probability that a bird in poultry house *i* dies or gets sick for any reason under normal conditions (i.e., when disease is not present).

p_{DC_i}: The probability that a bird in poultry house *i* dies or gets sick for any reason, including disease, under disease conditions (i.e., when disease is present).

[17] When this Appendix was written, it concentrated on dead birds only and did not consider sampling of "sick birds". However, "dead birds" may be substituted with "cull birds", defined as both dead and sick birds, hopefully without undue confusion or loss of generality.

p_i¢: The conditional probability that an animal dies or gets sick from the disease given that it has contracted the disease.

p_i: The prevalence of infection in the flock of house *i*, whenever disease is present.

$p_{Disease\text{-}\ dead_i}$: The probability that a bird in house *i* has the disease and dies from it. Or equivalently, this is the prevalence of infection among the population of dead birds in poultry house *i*.

a: Level of statistical significance. Note, that $(1-\ a)100\%$ is called the level of confidence.

g: The desired level of statistical confidence. This is equal to $(1-\ a)$.

n_i: Number of birds in house *i*[18].

$n_{dead\text{-}\ observed}$: Number of dead birds observed in a specified period of time, e.g., one week.

n_{random}: Minimum required number of birds to be sampled at random from the population of live birds (i.e., sample size) to find disease with $g\%$ statistical confidence.

$n_{targeted}$: Minimum number of birds to be sampled from the population of dead birds (i.e., sample size) to find disease with $g\%$ statistical confidence.

Note that the subscript *i* is used only to emphasize that different poultry houses in the same farm may have different mortality probabilities under normal and disease conditions. Since only one house will be discussed in this monograph, the subscript *i* will not be necessary and will be dropped from here on.

Fundamental assumptions for targeted sampling to be efficient

The fundamental assumption being made for targeted sampling of dead birds to be effective and more efficient than random sampling of live birds is that disease results in death that is statistically significantly higher than what would be expected under normal conditions. Otherwise, targeted sampling of dead birds would not be useful. The mathematical implication of this assumption is that the conditional probability p¢that a bird dies from disease given that it has contracted it must be greater than the probability that a bird dies from "natural causes" under no disease conditions. In other words, the number of dead birds must be greater under disease conditions than under normal conditions. This section will give a more formal mathematical argument for why this is so.

Define events as follows

Let *M* be the event that a bird dies for any reason, (i.e., the event of mortality), namely *M*={bird dies}. Let *D* be the event that a bird has the disease, (i.e., *D*={bird is diseased}). The conditional probability p¢that a bird dies from disease given that it has contracted it, and the prevalence p of disease in the flock can be expressed in terms of *M* and *D* as $p¢= P(M \mid D)$ and $p = P(D)$, respectively. By the total probability law,

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[18] Note that the total number of birds on farm is $N = \sum_{i=1}^{10} n_i$.

$$\begin{aligned} P(M) &= P(M \cap D) + P(M \cap \bar{D}) \\ &= P(M \mid D)P(D) + P(M \mid \bar{D})P(\bar{D}) \\ &= p' \times p + p_{NC}(1 - p). \end{aligned}$$

where p is the prevalence of disease in the flock and p_{NC} is the probability of death under normal conditions (i.e., when $p = 0.$) Thus, the probability of death (for any reason) is given by

$$P(M) = p' \times p + p_{NC}(1 - p).$$

The expected value of M (i.e., the number of birds expected to die for any reason, disease or otherwise) is $E(M) = n \times P(M) = n\left[p' \times p + p_{NC}(1 - p)\right]$ This is the same as the $E(Y_{DC})$, defined earlier as the number of birds expected to die under disease conditions, (i.e., for any reason, including disease). These two expected values are equal. That is, $E(M) = E(Y_{DC})$, and thus

$$E(Y_{DC}) = n\left[p' \times p + p_{NC}(1 - p)\right] \qquad (2)$$

The expected number of dead birds under disease conditions, $E(Y_{DC})$, is equal to the number of dead birds that have died of "normal causes", $E(X_{NC})$, plus those that have died from disease, $E(Z_{Disease-\ dead})$. That is

$$E(Y_{DC}) = E(X_{NC}) + E(Z_{Disease-\ dead}), \qquad (3)$$

where $E(X_{NC}) = n \times p_{NC}$. It follows that

$$\begin{aligned} E(Z_{Disease-\ dead}) &= E(Y_{DC}) - E(X_{NC}) \\ &= n\left[p' \times p + p_{NC}(1 - p)\right] - n \times p_{NC} \\ &= np' p + np_{NC} - np_{NC} p - np_{NC} \\ &= np' p - np_{NC} p \\ &= np(p' - p_{NC}). \end{aligned}$$

Thus,

$$E(Z_{Disease-\ dead}) = np(p' - p_{NC}). \qquad (4)$$

For $E(Z_{Disease-\ dead})$ to be positive, $p > 0,$ and p' must be strictly greater than p_{NC}. That is,

$$p' > p_{NC}. \qquad (5)$$

Thus, in order to have an increase in the number of dead birds due to disease, the condition in equation (5) must hold true. That is, the conditional probability p' that a bird dies from disease given that it has contracted it must be greater than the probability that a bird dies from "natural causes" under no disease conditions. It will be shown later in this monograph

(see Figures 1 and 2) that the relative efficiency of targeted sampling of dead birds to the random sampling of dead birds is inversely proportional to the difference *d* between $p¢$ and *p*. That is, relative efficiency $\mu \frac{1}{d} = \frac{1}{p¢ - p}$.

Estimating prevalence of disease in each population

Estimating prevalence of disease in the live-bird population

As stated in equation (3) above, when disease is present in the flock, the number of dead birds, denoted Y_{DC}, is the sum of the number of dead birds that have died from causes other than disease, denoted X_{NC}, and the number of birds that have died solely because of disease, denoted $Z_{Disease\text{-}dead}$. That is,

$$Y_{DC} = X_{NC} + Z_{Disease\text{-}dead} \qquad (6)$$

Each of these random variables has a binomial distribution. That is,

$$Y_{DC} : bin(n, p_{DC}); \quad X_{NC} : bin(n, p_{NC}); \quad \text{and} \quad Z_{Disease\text{-}dead} : bin(n, p_{disease\text{-}dead})$$

with expectations

$$E(Y_{DC}) = \hat{m}_{DC} = n \times p_{DC}$$
$$E(X_{NC}) = \hat{m}_{NC} = n \times p_{NC}$$
$$E(Z_{Disease\text{-}dead}) = \hat{m}_{Disease\text{-}dead} = n \times p_{Disease\text{-}dead},$$

respectively.

By the multiplication rule of probability, the probability $p_{Disease\text{-}dead}$ that a bird has infection *and* dies from it is equal to the conditional probability that a bird dies from infection given that it has infection (i.e., $p¢$), times the probability of infection (i.e., *p*). That is,

$$p_{Disease\text{-}dead} = p¢ \times p \qquad (7)$$

Since the expected value of the sum is equal to the sum of the expected values, it follows from equation (6) that $\hat{m}_{DC} = \hat{m}_{NC} + \hat{m}_{Disease\text{-}dead}$, and hence,

$$\hat{m}_{DC} = \hat{m}_{NC} + \hat{m}_{Disease\text{-}dead}$$
$$n \times p_{DC} = n \times p_{NC} + n \times p_{Disease\text{-}dead}$$
$$n \times p_{DC} = n \times p_{NC} + n(p \times p¢)$$

Dividing both sides by *n* and solving for *p* gives

$$p = \frac{p_{DC} - p_{NC}}{p¢}, \quad \text{for} \quad p_{DC} \geq p_{NC}, \qquad (8)$$

where $p \geq 0$ is the unknown disease prevalence in the flock, if it exists[19].

Equation (8) shows that the prevalence *p* of infection in the flock (i.e., the prevalence in the population of live birds) is the difference between the probabilities of death under disease

[19] Note that $p = 0$ under normal conditions (i.e., when disease is not present), and $p > 0$ (strictly greater than zero) when disease is present (i.e., under disease conditions).

conditions and normal conditions, weighted by (i.e., divided by) the conditional probability of death from disease given disease[20].

The prevalence p can be determined from knowledge about the probability of death under normal conditions p_{NC}, the conditional probability of death given infection, $p¢$, and the probability of death under disease conditions p_{DC}. Two of the three probabilities on the right hand side of equation (8), namely p_{NC} and $p¢$, are known or may be easily determined from historical records and from knowledge about the disease. The probability of death under normal conditions (p_{NC}) may be easily estimated from data collected in the particular poultry house under consideration on the number of birds that die under normal conditions by dividing the number of birds dying ($n_{dead\text{-}\ observed}$)in a given poultry house during a specified time interval, e.g., one week, by the total number of birds in that house (n). And the conditional probability that a bird dies from infection given it is infected ($p¢$) may be easily obtained from the literature and from knowledge about the disease death rate[21].

It will be shown in the next section that the probability of death under disease condition (p_{DC}) is equal to $n_{dead\text{-}\ observed} / n$. Substituting into equation (8) above gives the following equation for estimating p

$$p = \frac{[(n_{dead\text{-}\ observed} / n) - p_{NC}]}{p¢}. \qquad (9)$$

That is, the prevalence of disease in the flock (i.e., in the live-bird population) can be estimated as shown in equation (9)

Estimating probability of death under disease conditions

When disease is indeed present, its prevalence $p > 0$ (i.e., p must be strictly positive). And since $\hat{m}_{DC} > \hat{m}_{NC}$ Þ $n \times p_{DC} > n \times p_{NC}$, it follows that $p_{DC} > p_{NC}$. That is, when $p > 0$, p_{DC} is strictly greater than p_{NC}. As previously mentioned, $p_{DC} = p_{NC}$ only when $p = 0$.

The probability of death under disease conditions (p_{DC}) is estimated as follows.

$$\begin{aligned} E(Y_{DC}) - E(X_{NC}) &= \hat{m}_{DC} - \hat{m}_{NC} \\ &= n \times p_{DC} - n \times p_{NC} \\ &= n(p_{DC} - p_{NC}). \end{aligned}$$

Dividing both sides by n and solving for p_{DC} gives

$$\begin{aligned} p_{DC} &= \frac{E(Y_{DC})}{n} - \frac{E(X_{NC})}{n} + p_{NC} \\ &= \frac{E(Y_{DC})}{n} - p_{NC} + p_{NC} \qquad (10) \\ &= \frac{E(Y_{DC})}{n} = \frac{n_{dead\text{-}\ observed}}{n}, \quad \text{whenever } n_{dead\text{-}\ observed} > UCL_{NC}. \end{aligned}$$

[20] Note that p_{DC} ³ p_{NC} with equality if and only if $p = 0$ (i.e., when there is no disease in the flock).

[21] Note that $p¢$may be in the vicinity of 90% in unvaccinated flocks and around 2% in vaccinated ones.

To justify the last line of Equation (10), recall that the quantity in the numerator of equation (10), namely $E(Y_{DC})$, is the expected number of birds to die under disease conditions. The observed number of dead birds ($n_{dead\text{-}observed}$) represents either $E(Y_{DC})$ or $E(X_{DC})$ according to whether or not $n_{dead\text{-}observed}$ exceeds the predetermined upper confidence limit of the expected number of birds to die under normal conditions (UCL_{NC}). That is

$$n_{dead\text{-}observed} = \begin{cases} E(Y_{DC}), & \text{if } n_{dead\text{-}observed} > (1-\alpha)100\%UCL_{NC} \\ E(X_{NC}), & \text{if } n_{dead\text{-}observed} \le (1-\alpha)100\%UCL_{NC} \end{cases} \qquad (11)$$

Since the number of dead birds $n_{dead\text{-}observed}$ is expected to exceed the $(1-\alpha)100\%$ predetermined upper confidence limits under disease conditions, it follows that

$$p_{DC} = \begin{cases} \dfrac{n_{dead\text{-}observe}}{n}, & \text{if } n_{dead\text{-}observed} > (1-\alpha)100\%UCL_{NC} \\ 0, & \text{Otherwise} \end{cases} \qquad (12)$$

and hence

$$E(Y_{DC}) = n \times p_{DC} = n \times \frac{n_{dead\text{-}observed}}{n} = n_{dead\text{-}observed} \qquad (13)$$

Substituting $n_{dead\text{-}observed}$ for $E(Y_{DC})$ in equation (8) gives equation (9). Thus, equation (9) gives an expression for estimating the prevalence *p* of disease in the flock.

Estimating prevalence of disease in the dead-bird population

By definition, the prevalence of disease in a population is the number of birds with infection divided by the total number of birds in that population. For the population of dead birds, this is equal to the number of dead birds with the infection (i.e., $n_{Disease\text{-}dead}$) divided by the total number of dead birds ($n_{dead\text{-}observed}$). An infected dead bird is one that has contracted the disease *and* has died from it or due to it[22]. The number of dead birds that have died potentially from disease infection ($n_{Disease\text{-}dead}$) is equal to the number of dead birds observed ($n_{dead\text{-}observed}$) minus the number of birds expected to die under normal conditions ($E(X_{NC}) = n \times p_{NC}$) multiplied by the conditional probability of death given infection ($p\text{¢}$). Dividing the resulting number by the total number of dead birds in the dead-bird population (i.e., $n_{dead\text{-}observed}$) gives the desired estimate of the prevalence of infection in the population of dead birds. That is,

$$p_{Disease\text{-}dead} = \frac{[n_{dead\text{-}observed} - n \times p_{NC}] \times p\text{¢}}{n_{dead\text{-}observed}} \qquad (14)$$

[22] Note that it is possible that an infected dead bird might have died for reasons other than disease and not related to it. For END, however, this is not probable since it is much more likely that an infected bird will die from the disease than from other unrelated causes. Thus, if an infected bird dies, it will be assumed here that it has died because of infection.

The rationale behind this estimate is as follows. Out of the increased number of dead birds (i.e., $n_{dead\text{-}observed} - E(X_{NC})$), only a fraction of them (i.e., equal to $p\phi[n_{dead\text{-}observed} - E(X_{NC})]$) is expected to have died from the disease. Dividing this number by the total number of dead birds observed (i.e., $n_{dead\text{-}observed}$) gives the prevalence of disease in the population of dead birds, as given in equation (14).

To recap, the probability of mortality is estimated as $P(M) = n_{dead\text{-}observed} / n$ where $n_{dead\text{-}observed}$ is the number of dead birds observed and n is the total number of birds in the flock. If $n_{dead\text{-}observed} > UCL_{NC}$, then $n_{dead\text{-}observed} / n$ is the estimate of death under possible disease conditions. Otherwise, $n_{dead\text{-}observed} / n$ is the estimate of death under normal conditions. When $n_{dead\text{-}observed} > UCL_{NC}$, that is, when the number of dead birds observed exceeds the upper $(1-a)100\%$ confidence limits under normal conditions (recall UCL_{NC} is already set up and known), then the number of death increase (on average) due to disease can be estimated as $d = n_{dead\text{-}observed} - n_{NC}$[23]. If the conditional probability of death of an animal given that it has disease is $p\phi$ then the true number of diseased animals in the flock is estimated (on average) as $d / p\phi = (n_{dead\text{-}observed} - n_{NC}) / p\phi$ The prevalence of infection in the population of dead birds can be estimated (on average) as $(n_{dead\text{-}observed} - n_{NC}) / n_{dead\text{-}observed}$. Similarly, the prevalence of infection in the population of live birds can be estimated (on average) as $(n_{dead\text{-}observed} - n_{NC}) / n$. More conservative estimates for the prevalence of infection in the dead and live populations would be $(n_{dead\text{-}observed} - UCL_{NC}) / n_{dead\text{-}observed}$ and $(n_{dead\text{-}observed} - UCL_{NC}) / n$, respectively.

Having estimated disease prevalence in each of the two populations separately, the minimum number of birds that must be sampled from each population to detect disease at the $g = (1-a)100\%$ level of statistical confidence can now be determined. This is done in the following section.

Minimum sample size required to find disease in each population.

Population of live birds. Typically a poultry house contains a large number of birds. Thus, for the purpose of determining the minimum sample size necessary to swab in order to detect infection, the flock will be assumed to be large (e.g., 1000 or more birds). The minimum number of animals that must be sampled at random from the population of live birds assuming a disease prevalence p is given by [11]

$$n_{\text{random}} \approx \frac{\ln(a)}{\ln(1-p)}. \qquad (15)$$

[23] Another more conservative estimate of the increase in the number of deaths due to disease is $d\phi = n_{dead\text{-}observed} - UCL_{NC}$.

Population of dead birds. The minimum sample size required to find disease in the population of dead birds is calculated through the hypergeometric distribution. Unlike the large population of live birds above, the population of dead birds is a finite population that can be described with a hypergeometric distribution with a total number of $M = n_{dead\text{-}\ observed}$ dead birds, consisting of $D = p \times [n_{dead\text{-}\ observed} - E(X_{NC})]$ diseased birds, and $M - D = n_{dead\text{-}\ observed} - p \times [n_{dead\text{-}\ observed} - E(X_{NC})]$ non-diseased birds.

Recall that if the random variable *X* is the number of dead birds in a sample of size *n* taken from this population, then the probability distribution of *X* is the hypergeometric distribution given by

$$P(X = x) = \frac{\binom{D}{x}\binom{M - D}{n - x}}{\binom{M}{n}}, \quad 0 \le x \le n, \ x \le D, \ n \le M.$$

The minimum sample size required to detect disease in the population of dead birds ($n_{targeted}$) can now be determined accordingly by trial and error (see accompanying Excel spreadsheet model).

Forming Confidence Limits for Monitoring Dead Birds

Assuming that the probability p_{NC} of mortality under normal conditions is constant and that the *n* birds are independent with respect to their mortality under the same conditions, then the weekly number of birds (X_{NC}) dying under normal conditions can be modeled as a binomial random variable with parameters *n* and p_{NC}. The mean and variance of X_{NC} are $\mathrm{E}(X_{NC}) = n \cdot p_{\mathrm{NC}}$ and $\sigma^2_{X_{NC}} = np_{\mathrm{NC}}(1 - p_{\mathrm{NC}})$, respectively. That is, the number of birds expected to die for any reason per week in each house on average under normal conditions is $\mathrm{E}(X_{NC}) = \hat{\mu}_{\mathrm{NC}} = n \cdot p_{\mathrm{NC}}$. Its corresponding $(1 - a)100\%$ (or $g100\%$) confidence interval is given by $(\hat{m}_{\mathrm{NC}} \pm z_{a/2}\hat{s}_{\hat{m}_{\mathrm{NC}}})$, where $z_{a/2}$ is the *z*-value from the standard normal distribution corresponding to the $(1 - a)100\%$ level of statistical confidence. For example, at the 95% confidence (i.e., for $a = 0.05$), $z_{a/2} = 1.96$, and the lower and upper 95% confidence limits for $\hat{m}_{\mathrm{NC}}$ are

$$\mathrm{LCL}_{\mathrm{NC}} = \hat{m}_{\mathrm{NC}} - 1.96\hat{s}_{\hat{m}_{\mathrm{NC}}} = np_{\mathrm{NC}} - 1.96\sqrt{np_{\mathrm{NC}}(1 - p_{\mathrm{NC}})}$$

$$\mathrm{UCL}_{\mathrm{NC}} = \hat{m}_{\mathrm{NC}} + 1.96\hat{s}_{\hat{m}_{\mathrm{NC}}} = np_{\mathrm{NC}} + 1.96\sqrt{np_{\mathrm{NC}}(1 - p_{\mathrm{NC}})}.$$

Once confidence limits are constructed, they can be used in monitoring the number of dead birds per week. For instance, if the number of dead birds observed in a given time interval exceeds the upper confidence limit, animal health officials may decide to investigate further by testing animals. They may test live birds, dead birds, or both.

A Numerical Example

This example demonstrates how to calculate the relative efficiency of the proposed targeted sampling of dead birds to the random sampling of live birds described in the current 9 CFR 94.6. In this hypothetical example, it is assumed that a medium-sized commercial poultry farm consisting of $h = 10$ poultry houses of different sizes is being surveyed for END. There are five input parameters needed to estimate the prevalence of disease, calculate the minimum sample sizes required and determine the relative efficiency of the two sampling methods. These parameters are assumed to have values as follows:

1. The total number of birds in the poultry house is $n = 60000$.
2. The probability of death under normal conditions (i.e., from causes other than disease) is $p_{NC} = 0.001$.
3. The conditional probability that an animal dies from the disease given that it has contracted the disease is $p¢= 0.50$.
4. The number of dead birds observed in a given time period of interest is $n_{dead\text{-}\ observed} = 150$.
5. The statistical level of confidence is $g = (1- a) = 0.95$.

Estimated number of dead birds under normal conditions

The number of birds expected to die for any reason every week under normal conditions (i.e., other than disease since disease is not present under normal conditions) is:

$$\hat{\mu}_{\text{NC}} = np_{\text{NC}} = (60000)(0.001) = 60.$$

The associated 95% lower and upper confidence limits are:

$$\text{LCL}_{\text{NC}} = \hat{m}_{\text{NC}} - 1.96\hat{s}_{\hat{m}_{\text{NC}}} = np_{\text{NC}} - 1.96\sqrt{np_{\text{NC}}(1- p_{\text{NC}})} = 60 - 1.96\sqrt{60(1- 0.001)} = 45$$

and

$$\text{UCL}_{\text{NC}} = \hat{m}_{\text{NC}} + 1.96\hat{s}_{\hat{m}_{\text{NC}}} = np_{\text{NC}} + 1.96\sqrt{np_{\text{NC}}(1- p_{\text{NC}})} = 60 + 1.96\sqrt{60(1- 0.001)} = 76.$$

Expressed in terms of confidence intervals, the 95% confidence interval for the mean number of birds expected to die per week is (45, 76). This means that with 95% statistical confidence, the number of birds expected to die per week in poultry house one on average will be around 60 with lower and upper limits of 45 and 76, respectively.

The upper 95% confidence limit of 76 can now be used in monitoring the number of dead birds. For example, a decision to investigate further – perhaps by testing birds - to determine whether or not the increase is due to disease if the weekly number of dead birds exceeds the upper 95% confidence limit of 76. This would be justified since the increase is statistically significant.

Since the number of dead birds of $n_{dead\text{-}\ observed} = 150$ observed this week exceeds the predetermined upper confidence limit of 76, it may be concluded with 95% statistical confidence that there may be infection, or that there are irregularities in the flock causing a significant increase in mortality. Note that this conclusion is made before any sampling or testing of birds has been conducted.

Estimated probability of death under disease conditions

From equation (9), the probability of death under disease conditions, assuming possible infection, is estimated to be

$$\begin{aligned} p_{DC} &= \frac{n_{dead\text{-}\ observed}}{n} \\ &= \frac{150}{60000} \\ &= 0.0025. \end{aligned}$$

Thus, the probability of death under disease conditions is estimated to be $p_{DC} = 0.0025$. This is the rate of death for any cause, disease or otherwise and not just disease, when disease is present. Comparing it with $p_{NC} = 0.001$(i.e., the rate of death under normal conditions when disease is *not* present), this is a 150% increase over the rate of death under normal conditions (i.e., (0.0025-0.001)/0.001).

Estimated prevalence in the population of live birds

Substituting into equation (6) gives

$$\begin{aligned} p &= \frac{p_{DC} - p_{NC}}{p¢} \\ &= \frac{0.0025 - 0.001}{0.5} \\ &= 0.003. \end{aligned}$$

That is, the prevalence p of infection in the flock is equal to 0.003. Thus, it can be concluded with 95% statistical confidence that disease may be circulating in the flock with a prevalence of $p = 0.003$. This represents the prevalence of disease in the population of live birds.

Estimated prevalence in the population of dead birds

To estimate the prevalence of disease in the population of dead birds, we have by equation (11) that

$$\begin{aligned} p_{Disease\text{-}dead} &= \frac{[n_{dead\text{-}\ observed} - E(X_{NC})] \times p¢}{150} \\ &= \frac{[150 - 60] \times p¢}{150} \\ &= \frac{[150 - 60] \times (0.5)}{150} \\ &= \frac{(90) \times (0.5)}{150} \\ &= 45/30 \\ &= 0.30. \end{aligned}$$

That is, the prevalence of disease in the population of dead birds is estimated to be equal to

45/150=0.3 (or 30%).

The estimated prevalence of disease in the population of dead birds (i.e., $p_{Disease\text{-}dead} = 0.30$) is 100 times higher than the estimated prevalence in the population of live birds (i.e., $p = 0.003$).

Minimum sample sizes to detect disease

For the population of live birds

It follows from equation (15) that the minimum number of birds that must be sampled at random from the live-bird population to detect infection assuming the prevalence of infection in the flock is equal to $p = 0.003$ is

$$n_{\text{random}}{}^{3} \quad \frac{\ln(a)}{\ln(1- p)} = \frac{\ln(0.05)}{\ln(1- 0.003)} = 998.$$

Thus, detecting infection in the population of live birds with 95% confidence would require sampling at random at least $n_{random} = 998$ live birds.

For the population of dead birds

Similarly, with *M*=150, *D*=45, and $g = 0.95,$ the minimum sample size required to detect disease in the population of dead birds is equal to $n_{dead} = 8,$ as determined by trial and error using the hypergeometric distribution.

Thus, detecting infection in the population of dead birds with 95% confidence would require sampling at least $n_{dead} = 8$ dead birds.

Estimated relative efficiency

Finally, with $n_{random} = 998,$ and $n_{dead} = 8,$ the relative efficiency of sampling dead birds over random sampling of live birds given the parameter values in this example is

$$\begin{aligned} \text{Relative Efficiency} &= \frac{n_{random}}{n_{dead}} \\ &= 998/8 = 124.75 \\ &; \ 125 \ (\text{or } 12,500\%) \end{aligned}$$

Therefore, targeted sampling of dead birds is around 125 (12,500%) times more efficient than random sampling of live birds.

The relative efficiency decreases as the prevalence of infection in the flock increases. This is expected since, as the prevalence of infection increases its detection becomes easier with both methods. The graph shows that the lower the infection is the greater the relative efficiency of targeted over random sampling. Similarly, the effectiveness of targeted sampling in detecting infection increases as the prevalence of infection increases. This is also true for random sampling but the relative efficiency remains large between the two

methods for any prevalence.

Figure 1 shows how the relative efficiency changes with different values of n_{dead}, and $p¢$ The relative efficiency decreases as the number of dead birds increases, presumably as a result of a higher prevalence of infection in the flock. As stated above, the higher prevalence is, the easier to detect disease with either method.

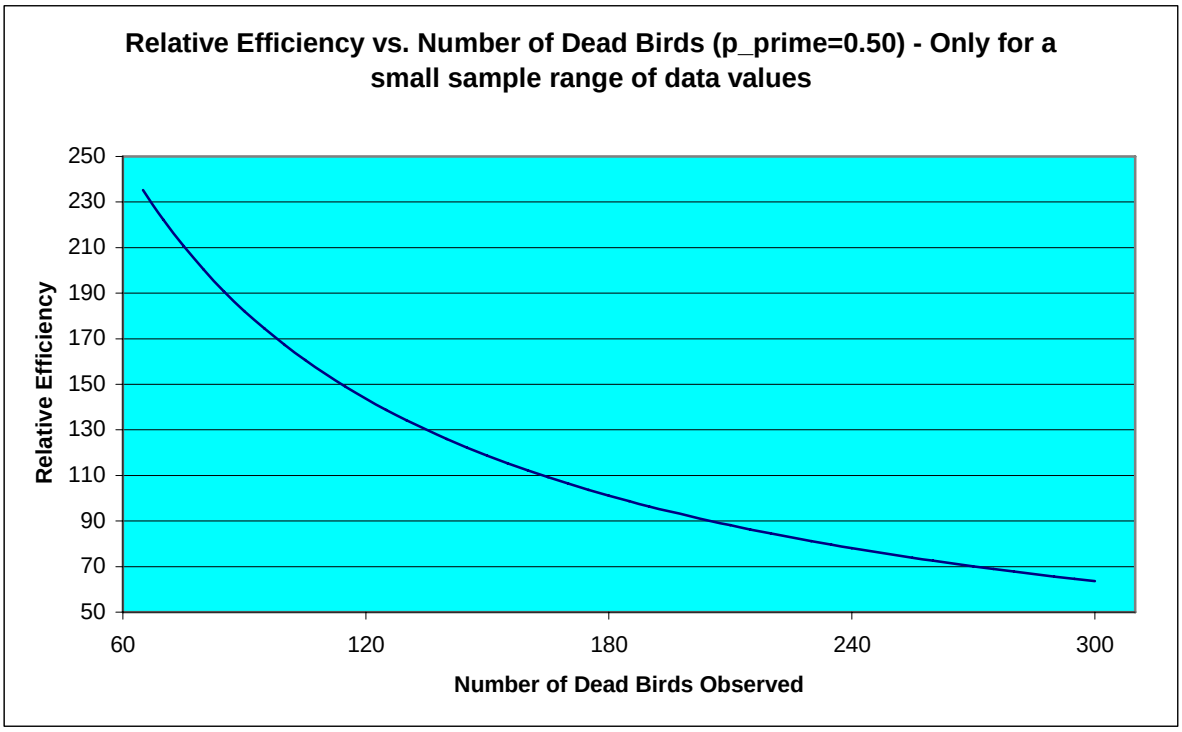

Figure 1. Relative efficiency of targeted sampling of dead birds to random sampling of live birds to detect END in a flock of size n=60000 at the 95% level of statistical confidence, assuming a rate of death under normal conditions $p_{NC} = 0.001$ and a conditional probability of death given disease $p¢= 0.50$.

APPENDIX C

CURRENT 9 CFR 94.6:

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(ix) And, if the eggs were laid in any region where END is considered to exist (see paragraph (a) of this section):

(A) No END occurred on the premises of origin or on adjoining premises during the 90 days before the certificate was signed.

(B) There is no evidence that the flock of origin was exposed to END during the 90 days before the certificate was signed.

(C) The eggs are from a flock of origin found free of END in one of the following ways:

(1) Sentinel birds \7\ were present in the flock of origin for at least 60 days before the certificate was signed. There was at least 1 sentinel bird per 1,000 poultry, with at least 30 sentinel birds per house. The sentinel birds remained free of clinical and immunological evidence of END as demonstrated by negative hemagglutination inhibition tests conducted on blood samples drawn at 10-day intervals by a salaried veterinary officer of the national government of the region of origin. The tests were conducted in a laboratory located in the region of origin, and the laboratory was approved to conduct the tests by the national government of that region or;

(2) Once every week, beginning at least 60 days before the certificate was signed, a salaried veterinary officer of the national government of the region of origin collected carcasses of all poultry that died during that week, and the carcasses were examined for END using the embryonated egg inoculation technique. Once a month, beginning at least 60 days before the certificate was signed, a salaried veterinary officer of the national government of the region of origin collected tracheal and cloacal swabs from not less than 10 percent of the poultry in the flock, and the swabs were tested for END. All examinations and tests were conducted in a laboratory located in the region of origin, and the laboratory was approved to conduct the tests and examinations by the national government of that region. All results were negative for VVND.