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Serological and sub-atomic investigation of Newcastle malady infection flowing in rural chickens of Fars area, Iran

Anastasia Audrey, Demir Efe, Alan Alexander

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Molecular Diagnostic Department, Central Veterinary Laboratory, Tehran, Iran.

Abstract

The epidemiology of circulating Newcastle disease virus in village chickens, using hemagglutination inhibition, RT-PCR and real-time RT-PCR was conducted by using systematic random sampling design for collection of samples. Total 1050 sera samples and 1820 tracheal and cloacal swabs were tested from chickens in 21 villages in Fars province, Iran. Samples were collected in January and February 2010. Hemagglutination inhibition test was used to screen the collected sera. RT-PCR and real time RT-PCR tests were used for virus detection in tracheal and cloacal swabs. The performed survey showed that chickens in thirteen (61.9%) villages (epidemiological units) were sero-positive, but no virus was detected in RT-PCR tests. Hemagglutination inhibition antibody titer varied from nil to 2^8 in the chicken sera. The chicken in the studied area were not vaccinated against Newcastle disease virus, but some of them showed high antibody titer (up to 2^8). This study shows that a pathogenic Newcastle disease virus is circulating in the area and it could be regarded as a potential threat to poultry industry at the studied area.

Keywords: Newcastle disease, village chickens, RT-PCR, real-time RT-PCR

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INTRODUCTION

Newcastle disease (ND) is a serious and commonly fatal viral poultry disease, which is present all over the world. In many tropical and subtropical countries virulent strains of ND virus (NDV) are endemic (Spradbrow, 1990). In most developing countries, ND is the most important infectious disease affecting village chickens (Spradbrow, 1999; Aini, 1990) and it causes great economic losses (Supramaniam, 1988). The causative agent is an enveloped, roughly spherical virus that is classified into the Paramyxovirus genus and the Paramyxoviridae family of the order mononegavirales. Correspondingly, the genome is a single-stranded negative-sense RNA including a gene sequence of 15186 nucleotides that code for six major proteins. The two glycoproteins haemagglutinin-neuraminidase (HN) and fusion protein (F) are exposed as protrusions on the surface of the virion envelope and are required to initiate viral infection. The HN protein is responsible for attachment of virus particles to sialic acid-containing receptors on host cells. The F glycoprotein is known as a biologic innate factor responsible for fusion between the virus and the cellular membrane, and so subsequent virus genome penetration. Moreover, the presence of multiple basic amino acids at the cleavage site of this protein indicates that the virus isolated is pathogenic (Alexander and Senne, 2008). Based on the severity of disease and the

clinical signs observed in infected chickens, the virus strains are divided into 5 pathogenic forms: (i) viscerotropic velogenic (ii) neurotropic velogenic (iii) mesogenic (iv) lentogenic (v) asymptomatic enteric (Beard and Hanson, 1984). There are several methods for pathotyping and characterization of NDV, such as intracerebral pathogenicity index (ICPI), intravenous pathogenicity index (IVPI) and mean death time (MDT) in SPF embryonated eggs (Alexander and Senne, 2008). Motivated by recent technological achievements in molecular modeling studies, different diagnostic techniques have been developed for detection and differentiation of NDV strains. Proposed by Office International des Epizooties (OIE) new regulations, reverse transcription polymerase chain reaction (RT-PCR) are applied in many laboratories of the world as the most reliable methods for the detection and identification of NDV. The tests have proved to be reliable in the detection and differentiation of virulent and non-virulent NDV and could be used as a robust test in veterinary diagnostic laboratories (OIE, 2000).

Virulent NDV strains have been isolated from all types of commercially reared poultry, ranging from pigeons to ostriches (Alexander et al., 2004). However chickens are the most susceptible avian species to the disease, waterfowl including geese and ducks are relatively resistant.

There can be little doubt that the severe form of ND is a serious problem either as an enzootic disease or as a cause of regular, frequent epizootics throughout some areas of Africa, Asia and Central and South America (Spradbrow, 1987; Spradbrow, 1988; Rweyemamu et al., 1991).

In Iran in addition to commercial poultry, native fowls are available. Native birds are considered a national asset and a key factor in creating sustainable agriculture in developing countries. These fowls provide a critical source of meat, egg, and income for people in rural areas and are operated predominantly by small and low income farmers. The government supports the native fowl production by providing a month old native chicks to the villagers (Rajabzadeh et al., 2010). A considerable population of domestic fowls and industrial intensive chicken farms exist in Fars region. No report has so far been published on the economical losses associated with NDV mortality in the rural areas. This study was carried out to detect the NDV infection and the patterns of spread in village chickens of Fars, using serological (HI) and molecular (RT-PCR) tests.

MATERIALS AND METHODS

Samples were collected in January and February 2010, throughout villages of the Fars provincial districts where the free range chickens are kept by the villagers. The systematic random sampling design was used for collection of samples. So, the samples were considered as representatives for the whole of the Fars provincial districts villages. Taking into account the number of villages where sampling was performed , that number was defined so as to ensure the identification of at least one infected holding if the prevalence of infected holdings is at least 5%, with a 95% confidence interval, where 21 out of 4340 villages were randomly selected and the number of free range sampled chickens from each villages was 50 birds so as to ensure 95% probability of identifying at least one positive bird if the prevalence of sero-positive birds is ≥ 30%. Totally 1050 sera samples were collected from chickens in 21 villages. For virus detection, seventy (70) tracheal swabs together with seventy (70) cloacal swabs were taken from apparently healthy free living birds of each selected villages. But the swabs were tested if the sera of the chickens in that village were positive in HI test. So, totally 1820 tracheal and cloacal swabs were screened by using RT-PCR test.

The chickens of the randomly selected villages were not vaccinated against Newcastle disease as stated by provincial veterinary officials. Specific care was taken for the storage and transport of samples. Swabs were chilled immediately on ice and submitted to the laboratory as quickly as possible. In the provincial central veterinary laboratory of Fars and Iran Veterinary Organization (IVO) laboratory sample processing and test performing were carried out in accordance with the Newcastle disease diagnostic manual (Commission Decision 92/66/EC) and OIE manual of standards for diagnostic test and vaccines (CEC, 1992).

For performing advanced tests tracheal and cloacal swabs were placed in a specific virus transport medium so that they were fully immersed, stored at 4°C and sent to the laboratory within 24 h. The transport medium used was COPAN virus transport medium (www.copaninnovation.com). Swabs were put in phosphate-buffered saline, immediately upon arrival at the laboratory, and after 1 h incubation at room temperature and centrifugation, supernatants were harvested. 0.2 ml of each swab buffer was taken and preparation of a mixture of seven swab buffer is done by adding 0.2 ml of each swab (totally 1.4 ml). 0.2 ml of this mixture used as final sample and RNA extraction followed by adding lysing buffer (0.4 ml). All the supernatants were pooled in batches of seven (each village considered as an epidemiological unit).

Extraction of viral RNA and RT-PCR

RNA was extracted from cloacal and tracheal swabs using commercial high pure RNA isolation kit (Roch, Germany) as recommended by the manufacturer.

Primers

A set of forward P1F (5′-TTGATGGCAGGCCTCTTGC-3′) and reverse P2R (5′-GGAGGATGTTGGCAGCATT-3′) primers were used according to oberdorfer and Werner (1998(. These primers flank the region encompassing the cleavage site of the fusion (F) protein. The expected size of PCR product was 362 bp.

Polymerase chain reaction (PCR)

PCR was carried out in a total volume of 25 μl containing 15 μl of H_2O, 2.5 μl of fast start buffer 10X, 1 μl of dNTPs 10 mM, 0.5 μl of

Probe: FAM APMV
5_-[FAM]TTCTCTAGCAGTGGGACAGCCTGC[TAMRA]-3_

Primer Forward APMV 1 F 5'-
AGTGATGTGCTCGGACCTTC-3'

Primer Reverse APMV 1 R 5'-
CCTGAGGAGAGGCATTTGCTA-3´

P1F (20 pmol/µl), 0.5 µl of P2R (20 pmol/µl), 0. 5 µl of RNasin (40 u), 0.5 µl of MMLV-Molony Murine Leukemia virus - (200 u), 0.5 µl of Fast start 5 u/µl and 5 µl of RNA. The thermocycler conditions were as follows:

40 min at 42°C, 7 min at 95°C, 30 S at 94°C followed by 55 cycles of 30 s at 53°C, 30 s at 72°C. we have already tested by one step RT- PCR method. The assays were performed on PTC200 MJ research system.

Detection of PCR products: PCR products were separated in 2% agarose gel 1× TBE buffer stained with ethidium bromide and visualized by ultraviolet (UV) transillumination. We also tested samples by Real time RT-PCR method (Creelan et al., 2002) using the primers and probe (FAM and TAMRA) for both Matrix gene (M gene) and Fusion gene as shown Table 1.

One-step real-time RT-PCR kits QuantiTect Probe RT-PCR kit (Qiagen GmbH, Hilden, Germany) was used. The reaction mixture for both assays contained 5 µl of RNA, 12.5 µl of 2× Buffer Mix, 900 nM of each primer and 0.25 µl of Taq Mix. In the M-gene and F gene assay, 250nM of M-probe was added. The RT-PCR thermal profile consisted of an initial RT step of 50°C for 30 min, followed by denaturation at 95°C for 15 min and 45 cycles of 20 s at 95°C and 60 s at 58°C in the F-gene assay and 52°C in the M gene assay. In order to test the RT-PCR efficiency, the La Sota strain RNAs extracted from the antigen solutions provided in house (CVL) were diluted serially 10-fold in sterile, nuclease-free water.

RNA extracted from Newcastle antigen (used in HI test) as positive control and was included in each test (Figure 1). Also some known positive samples from commercial chicken were tested. Our system could detect these samples. We got Ct about 20 and made standard curve by serial dilution 1:10 to check PCR efficiency. Efficiency was calculated about -3.3.

Serological procedure

The La Sota strain of NDV was used as the antigen for the HI test using 4 hemagglutinating units. Sera from all the birds were tested for the presence of HI antibodies against NDV antigen as described by van Boven et al. (2008). HI titers were regarded as being positive if there was inhibition at a serum dilution of 1/16 (2^4 or $\log 2^4$ when expressed as the reciprocal) or more against 4 hemagglutinating units of antigen (OIE, 2009).

RESULTS

In thirteen randomly selected epidemiological units (61.9%), ND HI antibody titer was from 2^4 to 2^8 as detected through HI test. In other 8 (38.1%) selected villages HI antibody titers were 2^3 or less than that ((Table 2 and Figure 2.). The geographical distribution of positive and negative epidemiological units is shown in Figure 3 (Map of Fars Province). As stated earlier, the village chickens in the selected area were not vaccinated against Newcastle disease. So, these finding shows that the ND viruses are circulating in the Fars provincial native chickens. However, the molecular performed tests (RT-PCR and real-time RT-PCR) did not detect the ND virus in the samples that were taken from seropositive chickens. Figure 1 shows that none of the test samples (from village chickens) were positive in this trial. Our PCR test was able to differentiate the positive control (La Sota vaccine strain) from negative control (Figure 1).

Antibody evidence of Newcastle disease virus infection was found on 13 of the 21 randomly selected surveyed villages free range chicken throughout all 4340 villages of the survey. The highest prevalence occurred in the Shiraz, Neiriz, and Mohr districts. Antibody titers were also high in the Khan Zenyan, Elyas Abad, Eslam Abad and Kuh mAreh Villages where serologically positive free range chickens were most prevalent.

DISCUSSION

Recently there has been increasing use of molecular techniques such as RT-PCR and real time RT-PCR to detect NDV in clinical specimens. The advantage of these tests is extremely rapid demonstration of the presence of virus (Alexander and Jones, 2008). These tests were employed in this experiment on the tracheal and cloacal swabs. But they could not detect the virus. Sensitivity of these methods was reported to be 93 to 100% (Śmietanka et al., 2006) and 95% (Alexander and Jones, 2008) when compared to virus isolation. This means that there was no active infection when the samples were taken.

According to the formal and official data no survey has been performed to show the apparent prevalence of Newcastle disease and or infection in village chickens in Iran. The examination of 1050 sera from unvaccinated village chickens revealed the presence of HI antibodies against NDV in 650 (61.9%) of the samples. In unvaccinated birds positive serology may be considered as diagnostic evidence of ND (Alexander and Jones, 2008). So, the presence of HI antibody against NDV indicates that these birds had contracted infection and recovered thereafter. In Iran there are about 62000 villages and every villager family is rearing these village chickens to produce meat and egg for their own

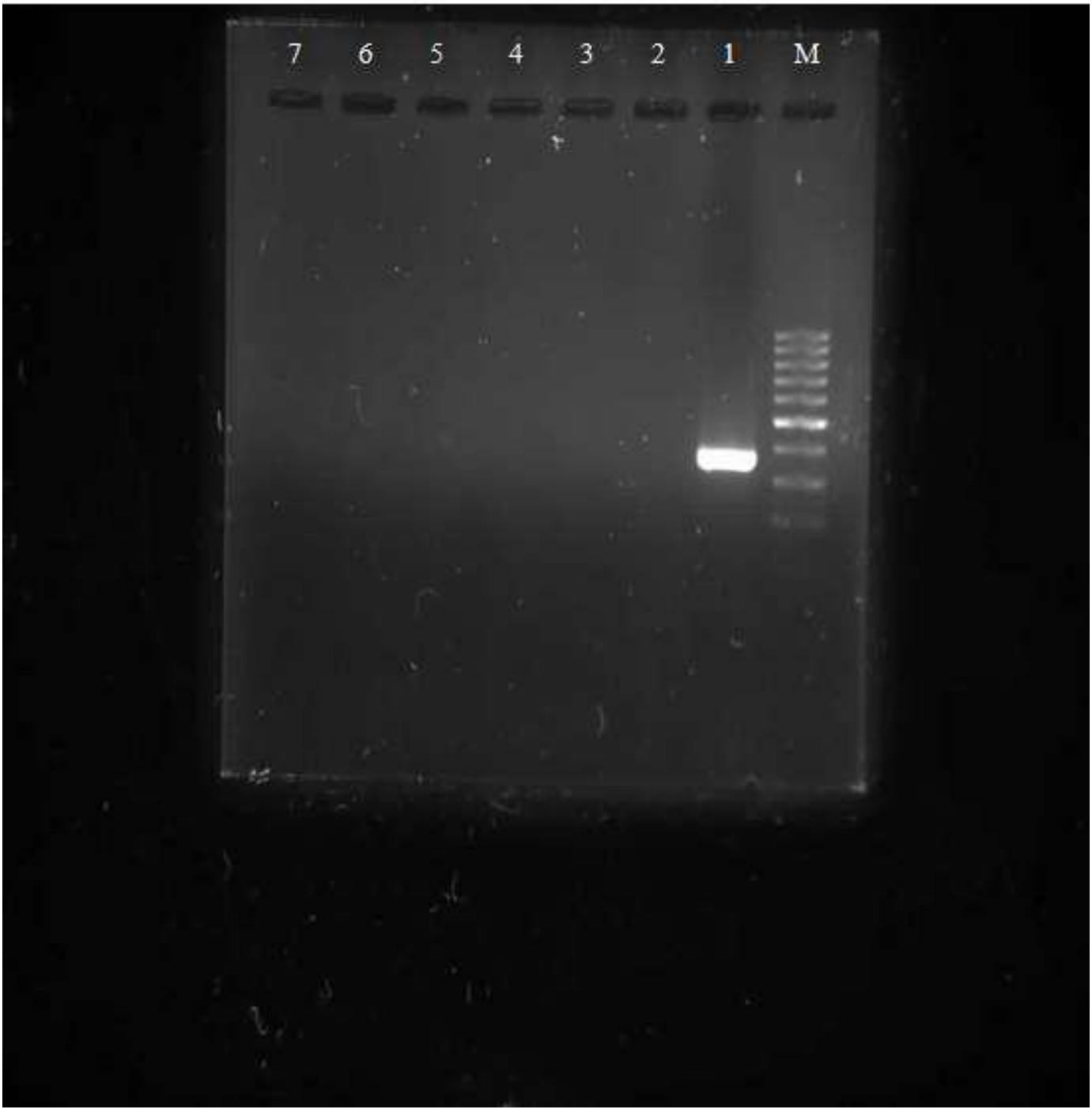

Figure 1. Result of the PCR assay: Amplifying 362-bp segment of F-gene of NDV. M: DNA (100 bp) marker, L1: positive control (LaSota vaccine strain), L2-L6: negative samples, L7: negative control.

consumption. These chickens are reared in a free range manner. On the other hand there are 20300 industrial chicken farms nearby these villages. Unvaccinated diseased village chickens constitute a potential risk in transmission of the virus to vaccinated commercial industrial chicken farms. This survey shows that the ND

Table 2. Maximum, minimum and mean HI antibody titer (log_2) of the chickens in the selected areas.

Selected area	Descriptive statistics				
	N	Minimum	Maximum	Mean	Std. deviation
Kohmare (SH)	50	0	8	3.84	2.411
Zangene (SH)	50	0	8	3.22	2.083
Khan Zenian (SH)	50	0	8	3.50	2.533
Zafarabad (SH)	50	0	2	0.62	0.830
Moharlu (SH)	50	0	7	3.54	2.169
Eliasabad (SH)	50	0	8	3.16	2.207
Bandeamir (M)	50	0	2	1.00	0.833
Dashtak (M)	50	0	2	0.66	0.717
Abgarm (M)	50	0	2	0.54	0.706
Hasanabad (M)	50	0	8	3.22	2.083
Karetabi (M)	50	0	7	2.62	1.937
Mohr	50	0	8	4.36	2.439
Ardevan (Mohr)	50	0	8	3.30	2.288
Juyan (Jahrom)	50	0	7	2.88	1.870
Dovlatabad (Neyriz)	50	0	7	2.68	2.004
Azigah (Neyriz)	50	0	7	2.76	2.066
Deris (Kaz)	50	0	8	2.98	2.208
Rashnabad (Kaz)	50	0	3	1.06	0.913
Sana (Jahrom)	50	0	2	0.76	0.771
Cheshmeraana (Eghlid)	50	0	2	0.42	0.702
Valid N (listwise)	50				

viruses are circulating in the Fars provincial free rang chickens where there may be temporary risk windows for introduction to Fars industrial poultry farms resulting in economic impacts for poultry industry sector. As there was no ND active infection, therefore the further survey should be conducted according to Susceptible Infectious Recovered (SIR) model.

One of the purposes of the survey was to determine the pattern of NDV spread in the village chickens of Fars Province molecularly, (virological survey). But according to the findings no positive molecular case was found. So the expression of the study is that according to this survey, molecularly we did not detect any patterns of NDV spread in the selected villages. But, serological finding implies that the ND viruses are circulating in the Fars provincial native chickens.

The potential of different pathotypes of NDV for stimulation of antibody production is different. Those viruses that are more virulent can produce more antibody titer. For example B1 strain may produce an HI antibody titer up to 2^4 and lentogenic strains can produce an HI antibody titer up to 2^6 (Dadras personal communication, unpublished). As we have shown in Table 2, some birds showed an HI antibody titer up to 2^8. This implies that mesogenic or lentogenic viruses have stimulated this antibody titer and these viruses have been regarded as pathogenic viruses. As stated earlier. "The chickens of the randomly selected villages were not vaccinated against Newcastle disease as stated by provincial veterinary officials."

In commercial birds ND is a problem in the Fars province. The disease has been observed frequently and the causative virus is detected and even registered in the gene bank (The Gen Bank accession number of the isolate of ND virus is JF820294 that is isolated from commercial broiler chicken). The village birds were apparently normal with no clinical sign of any illness at the time of sampling.

From the present study, it may be concluded that in order to control ND in Iran, vaccination of all poultry including village chickens should be made compulsory. Under no circumstance should commercial industerial chicken breeds be allowed to come into direct or indirect contact with village chickens.

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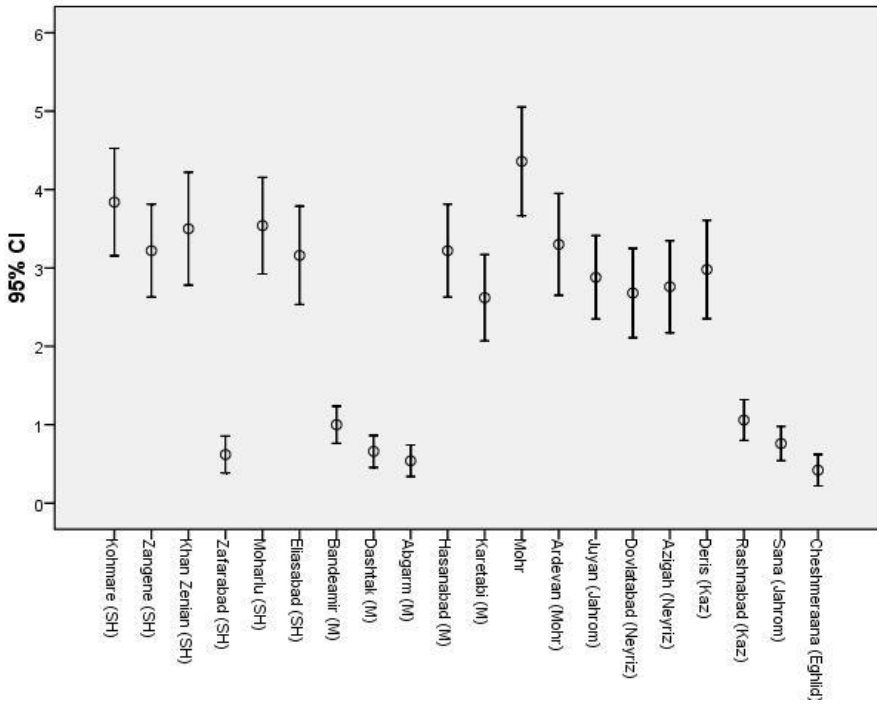

Figure 2. Distribution of mean of sera titers (log_2) and 95% confidence interval for each village sera samples. Values above 3 are considered as positive.

Figure 3. Map of Fars province showing the selected areas for sample collection. Dark circles show areas that the chickens had log2 HI antibody titer ≥4 and light circles show areas that the chickens had log2 HI antibody titer ≤4.

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