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Sample size of pooled sera for detection of chicken infectious bronchitis virus infection

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Abstract : The sample sizes required to detect at least one chicken infectious bronchitis virus (IBV) infection at flock-level were determined using pooled samples for 48 submissions with different samples in each. A total of serum samples of 9,980 layers from Kangwon, Chungpook and Chungnam province were collected and tested hemagglutination inhibition (HI) antibody titers against IBV both individually and with pooling size of 10. Of the 48 submissions, 72.9% were required less than 5 pools to detect at least one infected pool at 95% confidence level, and the corresponding rate was 77.1% at 90% confidence level. Overall, the number of pools was decreased as the percent of positive pools increased. At two different cut-off HI titer≥9 and≥10 for individual samples the seroprevalence was 50.1% and 33.4%, respectively while 59.9% were seropositive for pooled samples at HI titer≥8. The correlation coefficients between pooled and individual samples at each submission were 0.592 ($p<0.001$) for HI titer≥9 and 0.561 ($p<0.001$) for≥10, with common correlation coefficient of 0.576. This study indicated that pooled testing for the detection of IBV infection may be an alternative strategy when only the pooled results are of interest and the prevalence has not known exactly.

Key words **:** sample size, pooled serum, chicken infectious bronchitis, seroprevalence.

Introduction

In Korea, since the first report of infectious bronchitis virus (IBV) infection among laying hen breeders in 1986 further studies have also shown the presence of antibodies against IBV, indicating that the disease is widely spread throughout the country (18). Many researchers have assumed that the infectious bronchitis (IB) is prevalent in the majority of laying hen farms throughout the Korea with significant economic losses directly or indirectly, but the true prevalence in domestic laying populations has not been exactly known. For planning effective control strategies aiming at either control or eradication, it is necessary not only to determine prevalence of infection but also to diagnose infection at the flock or farm level in order to define the extent of infection. Of the serological diagnostic tests, antigen-detecting ELISA is the most rapid test to confirm the presence of IB virus (3). However, commercial kits are not widely available and diagnostic laboratories may need to source the reagents from the laboratories in which the monoclonal antibodies have been produced (7). Hemagglutination inhibition (HI) test remains the most commonly used for screening of IB antibody titers and under some circumstances may allow identification of infection as the test is rapid, inexpensive, and practicable (15).

Testing of pooled samples is a methodological strategy where samples from a number of individuals are aggregated into a single sample (pool), which is then tested for the disease or agent of interest. Particularly in a large population-based survey, testing of pooled sera from several individuals may offer a cost-effective alternative to testing individual serum samples. The pooled testing has also been used successfully in humans (9,12,14,17,19) and potential application in animals (2,4,5, 8,10,16). Epidemiological evidences showed that in the acute phase of an IBV infection of unprotected chickens, many birds produce large amounts of IBV in the trachea (3). However, in the case of chronic infections or infections in vaccinated birds, small amounts of virus may be present in only a low percentage of the birds, giving very low prevalence. In this context, appropriate sample size for detection of infected flock not individual birds is of more concern. Specific aim of this study was to determine sample sizes of pooled sera depending on the number of pools sampled for detection of IBV infection and to draw seroprevalence, which could give insights into the relevant researchers for planning economical IB surveillance programs.

Materials and Methods

Serum sample and serology

During 2003-2006, a total of 9,980 serum samples of laying flocks were collected for detection of IBV infection from the three provinces (Kangwon, Chungpook, Chungnam) of Korea. HI tests for pooled samples were done similar to the procedures described previously (11). For pooling, randomly combined samples at each submission (date of testing) were

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pooled into 998 pools of 10 samples each. These pools were constituted by transferring 100 μl from each of 10 consecutive samples. The antigens (DaeSung Microbiological Labs, Korea) were diluted to contain 4 hemagglutination units (HAU), and the titers were expressed as logarithmic scale (base 2) of the reciprocal of the highest dilution of serum giving 100% inhibition of 4 HAU. Two HI titers ≥ 10 or ≥ 9 for individual samples and HI titers ≥ 8 for pooled samples were considered seropositive.

Modeling of sample size

Sample size was simulated to determine the probability of at least one infected pool depending on the number of pools sampled. For each submission, the number of pools tested and the number of pools with seropositive were entered into an Excel spreadsheet (Microsoft®, Seattle, WA). Simulation was performed using the HYPGEOMDIST function, and the sensitivity and specificity of the HI test were assumed to 90% and 98%, respectively. Pearson correlation coefficients and combined common coefficients were computed using standard normal distribution.

Results

The average number of pools that were tested at each submission was 21, with a range of 6-43 (Table 1). Of a total of 9,980 individual serum samples, 5,000 (50.1%) and 3,336 (33.4%) were seropositive for HI titer ≥ 9 and ≥ 10, respectively. Of the 998 pooled samples, 598 (59.9%) were seropositive. To detect at least one infected pool for 48 submissions, 72.9% and 77.1% were required less than 5 pools at 95% and 90% confidence level, respectively. The number of pools was decreased as the percent of positive pools increased (Fig 1). When the observed prevalence of pooled samples were compared with those of individual samples at each submission, the correlation coefficients were 0.592 ($p < 0.001$) for HI titers ≥ 9 and 0.561 ($p < 0.001$) for titers ≥ 10 (Fig 2) with no significant difference between the two coefficients. The common correlation coefficient was 0.576.

Discussion

IBV infection is an individual chicken disease, but more importantly can be considered a flock problem. From a disease control perspective of view, early detection of infected flocks is essential to provide early intervention and to curb further spread the disease within flocks. This may lead to minimize economic loss caused by the infection. With this viewpoint, diagnostic objective can be set to test flocks of chickens to classify them as infected or non-infected. When the number of pools required for the detection of at least one infected pool was evaluated for 48 submissions, the number

Table 1. Number of pools for the detection of one or more positive pools at each test number (submission), assuming sensitivity of 90%, specificity of 98% and pooling size=10

Test no.	Pooled sample		No. of pools required at CL* of:		No. positive for individual sample	
	No. tested	No. positive	95%	90%	HI≥9	HI≥10
1	13	3	8	7	31	9
2	10	9	1	1	63	44
3	11	7	3	3	20	8
4	14	10	3	2	72	40
5	15	7	4	4	51	29
6	11	7	3	3	64	53
7	13	7	4	3	52	31
8	16	10	3	2	66	34
9	21	13	3	3	66	31
10	13	13	1	1	84	57
11	22	12	4	3	143	94
12	23	11	5	4	118	81
13	27	10	6	5	160	114
14	30	9	7	6	96	56
15	21	11	4	3	92	49
16	40	4	19	16	100	65
17	39	10	9	7	179	109
18	34	10	8	6	171	98
19	24	7	7	6	116	72
20	43	7	13	11	176	103

Table 1. Number of pools for the detection of one or more positive pools at each test number (submission), assuming sensitivity of 90%, specificity of 98% and pooling size=10. (Table 1 Continued)

Test no.	Pooled sample		No. of pools required at CL* of:		No. positive for individual sample	
	No. tested	No. positive	95%	90%	HI≥9	HI≥10
21	39	8	11	9	209	149
22	21	7	6	5	96	62
23	25	17	3	2	127	81
24	23	17	3	2	121	82
25	16	14	2	2	78	54
26	13	7	4	3	36	24
27	17	14	2	2	86	50
28	11	9	2	2	49	28
29	16	7	5	4	24	8
30	20	16	2	2	119	80
31	22	19	2	2	126	80
32	21	18	2	2	112	83
33	16	11	3	2	75	50
34	7	4	3	3	27	14
35	19	12	8	7	65	43
36	15	15	1	1	93	63
37	31	28	2	1	154	84
38	16	14	2	2	45	15
39	9	7	2	2	56	32
40	18	17	1	1	134	100
41	20	13	3	3	102	78
42	23	17	3	2	111	62
43	24	24	1	1	213	161
44	40	40	1	1	333	280
45	25	22	2	2	182	157
46	22	22	1	1	175	139
47	6	6	1	1	53	49
48	23	16	3	2	79	51
Total	998	598			5,000	3,336
Mean	21	12	4	3	104	70
Minimum	6	3	1	1	20	8
Maximum	43	40	19	16	333	208

*confidence level.

of pools showed decreasing trend as the percent of positive pools increased. This trend generally seems to be expected for all circumstances. However, all submissions in this study were from any flocks or farms regardless of infection status, resulting in relatively slow decreasing pattern. If it were collected only from infected flocks, it is expected to be more steeply decreased until some level of percent positive pool and slowly decreased thereafter. Assuming 30-60% of the pooled prevalence, 11 of 48 submissions was classified as this category. For 10 of these, sampling of 3-4 pools provided 95% confidence of detecting at least one infected pools, while for the remaining one submission (submission no. 5), sampling of 5 pools provided the same level of confidence.

The seroprevalence study of IBV infection is very limited. Gutierrez-Ruiz et al (6) reported that the seroprevalence was 56.5% with a range of 28.2-97.6% in free-range chickens of Yucatan, Mexico. In a survey performed on free-range chickens in California, 46.7% of 30 flocks examined and 21.8% of birds tested were positive for IBV antibodies by an ELISA test (13). The apparent seroprevalence in this study was 33.4-50.1% for individual samples and 59.9% for pooled sera. However, it is very cautious to consider this result as overall seroprevalence of IBV infection in laying flocks studied. Since samples in this study were from field veterinarians in private

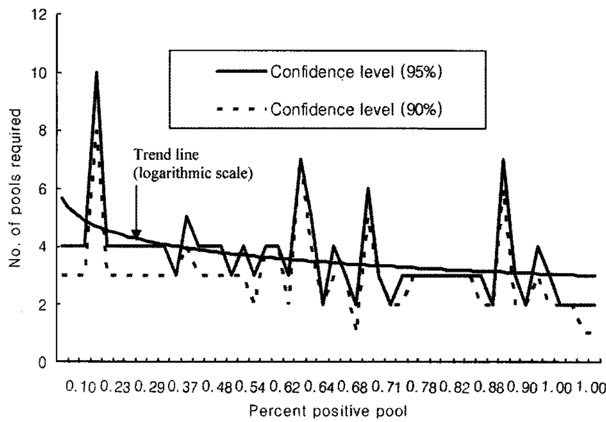

Fig 1. Simulated number of pools required to detect at least one positive pool. Assumption: sensitivity=90%, specificity=98%, pooling size=10.

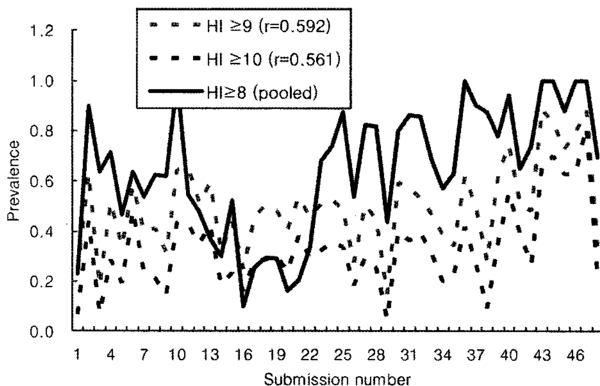

Fig 2. Comparison of prevalence between pooled and individual serum samples with different cut-off for seropositivity. r=correlation coefficient.

practice who are specifically aiming to consult for farm owners it would seem likely that submissions are convenience or purposive samples, resulting in biased prevalence: the higher proportion of samples from farms with low prevalence the lower the prevalence of IBV infection. If the seroprevalence in this study is considered to be significantly higher or lower than expected, several explanations may be addressed to the discrepancy. Most probable explanation is that only one serotype (Massachusetts) was used as antigen, although this serotype gives more cross-reactions than other serotypes (1). This might be supported by the results of an epidemiological study, in which the authors reported that at least four genetically and serological different variant serotypes of IBV were prevalent in Korea (18). Another explanation could be that although diagnostic cut-off used for classifying seropositive in individual and pooled samples were based on expert's experiences after considering vaccination practices in the field, those criteria are remains in controversial. Therefore, false negatives or false positives could be expected depending on the criteria. Together with this, in pooled testing situations as described earlier, it is critical that samples are selected and allocated to pools in a random manner to prevent clustering and resultant biasing of results. Lastly, drawbacks associated with pooled testing may also contributing factors: if the pooling size is large, some positive sera may be excessively diluted by negative sera and become undetectable in the pool, yielding prevalence underestimation. To deal with this problem and for estimating prevalence without identifying the infected samples, researchers proposed parametric procedures based on the hierarchical pooling model (21) or cut-off points that depend on the pooling size (20). The authors also suggested their approaches in use even if the prevalence is high. Any of these factors may have been affected in the prevalence.

The prevalence obtained from this study deserves consideration in the future. To enhance general applicability and value of pooled testing, determination of the number of samples to be pooled at various cut-off levels of seropositivity and sensitivity of the test with various antibody levels may be subjects of interest.

Acknowledgements

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닭 전염성 기관지염을 검출하기 위한 합병혈청의 표본크기

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요 약 : 계군 수준에서 닭 전염성 기관지염 (IBV)을 검출하는데 필요한 표본크기를 추정하기 위하여 강원도, 충북 및 충남 지역의 총 9,980수의 산란계로부터 회수된 총 48회의 혈청시료를 사용하였다. 의뢰된 모든 혈청에 대해서는 개별 시료와 크기가 10인 합병혈청 (pool)으로 구분하여 HI 역가를 측정하였다. 적어도 1개의 감염된 pool을 검출하는 것을 95% 신뢰하기 위해서는 총 48회의 의뢰건 중 5개 이하의 pool이 요구되는 비율이 72.9%를 차지하였고, 90% 신뢰수준에서는 77.1%로 나타났다. 전체적으로 볼 때 필요한 pool의 개수는 양성 pool의 개수가 증가할수록 감소하였다. 개별시료에서 양성판정을 위한 HI 역가의 기준을 9 이상과 10 이상으로 설정할 때 혈청 유병율은 각각 50.1%와 33.4%로 나타났으며, 합병혈청에 대한 양성 판정기준을 8 이상으로 설정할 경우 59.9%로 분석되었다. 매 의뢰된 시료에서 개별시료와 합병혈청 간의 상관계수는 판정기준 9 이상에서 0.592 ($p<0.001$), 10 이상에서 0.561 ($p<0.001$)로 두 상관계수간 차이가 없었고 공통상관계수는 0.576으로 나타났다. 이러한 결과에 근거할 때 IBV 감염증을 검출하기 위하여 합병혈청을 사용하는 전략은 조사의 목적이 계군의 감염여부에만 관심을 두는 경우 한가지 대안이 될 것으로 사료된다.

주요어 : 표본크기, 합병혈청, 닭 전염성 기관지염, 혈청유병율