

## Evaluation of five different antigens in enzyme-linked immunosorbent assay for the detection of avian pneumovirus antibodies

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**Abstract.** Five different antigens were evaluated in enzyme-linked immunosorbent assay (ELISA) tests for the detection of avian pneumovirus (APV) antibodies. Two of the 5 antigens were prepared from recent APV isolates from Minnesota. The 2 older isolates were passage 63 of a strain currently used as a live, attenuated vaccine and a Colorado strain isolated for the first time in the United States and currently used in an ELISA test. The fifth antigen is based on an APV recombinant N-protein. Basic parameters and positive–negative threshold of the assays were established for all 5 antigens on the basis of data obtained by testing 46 known negative and 46 known positive serum samples. Subsequently, 449 field samples were tested by all 5 ELISAs. The optical density difference (ODD) was calculated by subtracting optical density of the sample in the negative antigen well from that in the positive antigen well. In the current ELISA test based on the Colorado strain, an ODD of 0.2 is considered to be the cutoff value to classify samples as negative or positive. In this study, however, use of different cutoffs, based on ODD of negative control plus 3 SD or values estimated from Receiver operating characteristic analysis, was considered to be more appropriate for the various antigens used. Overall person-to-person and day-to-day variability was found to be large for all tests using either ODD or sample to positive ratio to report results. In addition, results suggest that antigenicity of the APV isolates in the United States has not changed between 1997 and 2000.

### Introduction

Avian pneumovirus (APV) causes an economically important upper respiratory tract infection of turkeys. The diagnosis of APV infection is accomplished by the demonstration of viral nucleic acid by reverse transcription–polymerase chain reaction,<sup>6,21</sup> virus isolation,<sup>9</sup> or serology. Serological testing is an important component of epidemiological surveillance and for the evaluation of vaccination strategies. Tests such as indirect fluorescent antibody, virus neutralization, and enzyme-linked immunosorbent assay (ELISA) have been used for the detection of anti-APV antibodies.<sup>1,2,11,14,16,23</sup> Of these, ELISA is considered to be a rapid and economical test and is widely used in Europe.<sup>2,8,11</sup> When APV first appeared in the United States in 1996, the ELISA tests based on European virus isolates were not able to detect antibodies to US isolates of APV but those using the US isolate of APV did.<sup>20</sup> The antigen used in the latter test was a whole-virus lysate of the Colorado isolate of APV (APV/Colorado/turkey/97) isolated in 1997 from turkeys in Colorado.<sup>3,10,20</sup>

Antigenic diversity in different APV isolates is well documented. On the basis of molecular sequencing of surface glycoproteins G and F, 2 subgroups (APV-A and APV-B) of APV have been identified in Europe. The US isolates of APV are distinct from the European subgroups and have been placed in subgroup C (APV-C).<sup>19</sup> The amino acid sequence of N-protein of APV-C has only 69% identity with those of APV-A and APV-B, whereas identity within APV-C isolates is 99.7%.<sup>5</sup> Variations in APV have also been evidenced serologically by cross-neutralization and by using monoclonal antibodies.<sup>4,13,15</sup> These antigenic differences have been shown to affect the sensitivity of the ELISA tests. For example, when birds vaccinated with attenuated vaccine prepared from French isolates were tested in an ELISA test using UK isolates, fewer samples were found to be positive than when homologous virus was used for testing.<sup>7,24</sup>

ELISA test for the detection of antibodies against APV-C was developed using the Colorado virus as an antigen.<sup>3</sup> It was recommended at that time that at least 10 samples from each suspect flock should be tested to determine the infection status of the flock. During the first 3 years of its use, this ELISA test invariably detected all 10 samples from a flock to be either negative or positive. More than 10,000 flocks of turkeys have been tested without any deviation from these results. Recently, however, ELISA-positive samples have been ranging from 1 to 10 per flock. Arbitrarily,

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**Table 1.** Standardization of antigen and conjugate dilutions for APV ELISA.\*

Antigen name	Virus name	Optimum dilution of antigen	Optimum dilution of conjugate
MN-16	APV/MN/turkey/16/2000	1:3,500	1:6,000
MN-18	APV/MN/turkey/18/2000	1:3,500	1:6,000
MN-1a (P63)	APV/MN/turkey/1-a/97	1:3,200	1:4,000
APV-CO	APV/Colorado/turkey/97	1:800	1:1,500
N-protein†	recombinant protein from MN-1a	1:200	1:1,500

\* Dilutions of antigen and conjugate were standardized using checkerboard titration with 1:40 dilution of known positive and known negative serum samples.

† Anti-N antibody was used at a dilution of 1:1,500.

farms with 4 or more positive samples are now considered positive in Minnesota. Because most of the samples being tested are from turkey-processing plants, the birds in the “suspect” category cannot be retested. Some believe that the virus currently circulating in Minnesota turkey flocks has changed and that this change may be responsible for the above-noted phenomenon. Also, APV (MN-1a) passaged serially in cell cultures for 63 passages (labeled as P63) has been used as a vaccine since 2001, which might have exerted selection pressure on the virus circulating in the turkey population. This study was therefore designed to compare the performance of 5 ELISA assays, each based on a different antigen, on experimental and field samples. The specific objectives of the study were to 1) evaluate the effectiveness of the 5 assays to differentiate between samples from infected and noninfected individuals, 2) examine the use of the assay on field samples, and 3) examine the variability of each assay.

### Materials and Methods

**Antigens.** Four whole-virus antigens and 1 APV recombinant protein were used as antigens. Of the 4 whole-virus antigens, 2 were prepared from recent isolates of APV. These viruses were isolated in 2000 from Ottertail and Kandiyohi counties of Minnesota and were adapted to grow on Vero cells for 9 passages (antigen 16 and antigen 18). The third antigen was prepared from the Colorado isolate of APV (antigen CO). This virus was originally isolated from turkeys in Colorado in 1997 and is currently used in the ELISA test in the United States.<sup>3</sup> The fourth antigen was prepared from passage 63 of APV/MN/turkey/1-a/97 (antigen P63), a strain used as commercial live, attenuated vaccine.<sup>17</sup> Recombinant N-protein of APV<sup>12</sup> expressed in *Escherichia coli* was used as the fifth antigen (antigen N).

**Serum samples.** A total of 541 serum samples were tested. Of these, 46 sera were from turkeys experimentally infected with a Minnesota isolate of APV (MN-2a) and 46 were from noninfected turkeys. The remaining 449 samples were field sera from 163 different flocks of turkeys selected randomly from a pool of serum samples available at the Minnesota Poultry Testing Laboratory, Willmar, Minnesota. True infection status of these randomly selected samples was unknown. To determine day-to-day and person-to-person variation, 46

samples were selected randomly from the 449 field samples and tested by ELISA using all 5 antigens. The samples were tested in duplicate at 2 different times by 2 different persons. The operators were blinded to the true status of the samples.

**Antigen preparation.** Viral antigens were prepared as described previously.<sup>3</sup> In brief, the viruses were propagated in Vero cells in 75-cm<sup>2</sup> flasks. After the development of syncytia in cell cultures, usually within 2–3 days, the cell culture fluid was decanted and the monolayer was washed. It was then treated with 0.5 ml of 0.5% vol/vol IGEPAL CA-630,<sup>a</sup> a nonionic detergent prepared in 0.01 M phosphate-buffered saline (PBS) (pH 7.2). The flask was placed on a rocking platform at 4 C for 1 hr. The resulting suspension was removed, centrifuged at 3,000 × *g* for 15 min at 4 C and used as antigen. Negative control antigen was prepared from mock-infected Vero cells treated in a manner similar to that for viral antigen. Optimal dilutions of the antigens and the conjugate were determined by checkerboard titration using positive and negative control sera at 1:40 dilution prepared in dilution-blocking buffer.<sup>b</sup>

**ELISA procedure.** Viral and negative control antigens were placed in alternate rows of an ELISA plate<sup>c</sup> at 100 μl per well followed by overnight incubation at 4 C. After washing with PBS containing 0.05% Tween 20 (PBST), the antigen-coated plates were stored filled with washing buffer at –20 C for further use. For use in the test, the plates were allowed to attain room temperature, washed 3 times with PBST, and blotted dry. Test sera were diluted 1:40 in dilution-blocking buffer<sup>b</sup> and added to both the antigen-coated well and the negative control well at 50 μl/well. Known positive and negative control sera were included in each plate. The plates were incubated at room temperature for 1 hr, washed, and blotted dry. The conjugate (antiturkey immunoglobulin G horseradish peroxidase)<sup>b</sup> was standardized for each antigen (Table 1), optimum dilutions of conjugate added at 50 μl per well, and incubated for 1 hr at room temperature. After washing, substrate solution consisting of 0.05 M citrate phosphate buffer (pH 5.0), 0.04% (wt/vol) O-phenylenediamine, and 0.04% (vol/vol) H<sub>2</sub>O<sub>2</sub> was added. Color development was stopped by the addition of 25 μl of 2.5 M sulfuric acid. The optical density was read at 490 nm (A<sub>490</sub>), and the results were expressed as optical density difference (ODD), the difference between the absorbance of APV antigen well and that of negative control well for each serum sample. A positive control was included on each plate. The corrected optical density (OD) of the positive control

**Table 2.** Mean ODD and SD of 46 known negative sera using 5 different antigens.\*

Antigen name	Virus name	Mean ODD $\pm$ SD
MN-16	APV/MN/turkey/16/2000	0.063 $\pm$ 0.013
MN-18	APV/MN/turkey/18/2000	0.064 $\pm$ 0.015
MN-1a (P63)	APV/MN/turkey/1-a/97	0.099 $\pm$ 0.029
APV-CO	APV/Colorado/turkey/97	0.097 $\pm$ 0.035
N-protein	N-protein	0.075 $\pm$ 0.019

\* All sera were tested at dilutions of 1:40.

was used as the denominator to calculate sample to positive (S/P) ratio of all samples.

**N-protein ELISA.** The plates for N-protein ELISA were prepared as described previously.<sup>12</sup> In brief, the plates were coated with 100  $\mu$ l per well of rabbit N-protein-specific antiserum and preimmune serum (positive and negative rows, respectively) diluted 1:1,500 in 0.05 M sodium carbonate-bicarbonate buffer (pH 9.6) followed by incubation at 37 C for 2 hr. The plates were blocked by the addition of 100  $\mu$ l of 4% fetal horse serum (in PBST) into each well followed by incubation at 4 C overnight. After washing, the plates were stored filled with washing buffer at  $-20$  C until use. The test procedure was the same as that for whole-virus antigen ELISA except that the first step after coating was addition of Ni-NTA column-purified N-protein at 1:200 dilution to all wells. The plates were incubated for 1 hr at room temperature, washed 3 times, and then 1:40 diluted serum samples were added.

**Data analysis.** On the basis of 92 experimental samples, the 5 tests were first evaluated by estimating the effect of using various cutoffs on the proportion of known positive and known negative samples that were correctly identified. Selection of the optimal cutoff for a given test was done using Receiver operating characteristic curves. ROC curves allowed the authors to compare the effectiveness of each assay at various cutoff points, where the optimal cutoff is the one that maximizes the proportion of known positive samples correctly classified (sensitivity) while minimizing the proportion of negative samples that are incorrectly identified as positive (specificity). The effect of using 3 different

approaches to identify cutoffs was examined on the set of experimental samples and of field samples of unknown status. The cutoffs used were 1) the traditionally used ODD of 0.2 applied to all 5 tests,<sup>2,3,8</sup> 2) the optimal cutoff identified for each test using the ROC curves, and 3) ODD of the negative serum samples for each test plus 3 SD. The proportion of samples that tested positive or negative was compared between tests at the various cutoffs.

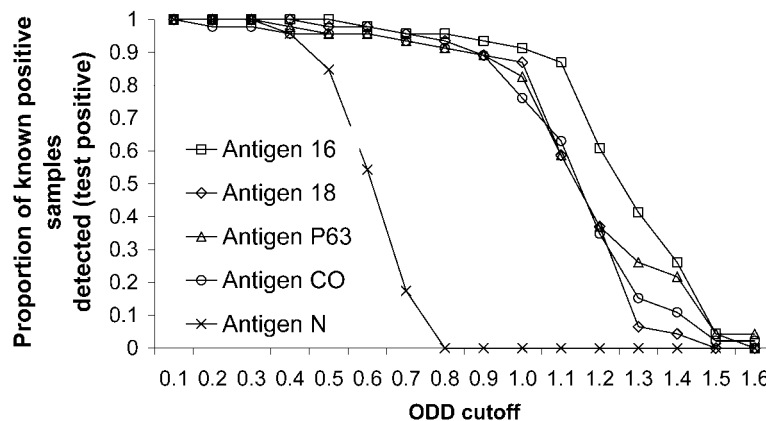
The effect of person-to-person and day-to-day variability for the 5 assays was evaluated based on comparison of coefficients of variations (CV). The CV for each of the 46 samples tested was calculated using the formula  $SD/mean$ , where mean and SD were obtained from the values of ODD from person 1 and person 2 at day 1 and day 2. Median (50th percentile), 25th and 75th percentile, and minimum and maximum CV for each test were obtained from the collection of CV for each of the 46 samples tested. A mixed-effects analysis of variance was used to evaluate the effects of person and day on the ODD results.<sup>18</sup>

## Results

**Standardization of ELISA.** The results of checkerboard titration for the 5 antigens and the conjugate are shown in Table 1. The optimum dilutions of antigens prepared from recent isolates of APV (antigens 16 and 18) and the vaccine virus (P63) were lower than those from antigens CO and N. The reason for higher dilutions used for these antigens may be because of their better adaptation to grow in Vero cells.

**Negative threshold.** Known negative sera from non-infected birds ( $n = 46$ ) were tested against all 5 antigens. Average ODD of all sera along with their standard deviations are shown in Table 2. The mean ODD ranged from 0.063 to 0.099 depending on the antigen used. These values were used to compute the threshold of negative sera for each antigen.

**Evaluation of the assays on experimental samples.** All assays detected all known positive samples using an ODD cutoff value of 0.1 or 0.2, except for antigen CO, where only 45 of the 46 (98%) positive samples



**Figure 1.** Proportion of known positive samples that tested positive for a given ODD cutoff for each assay.

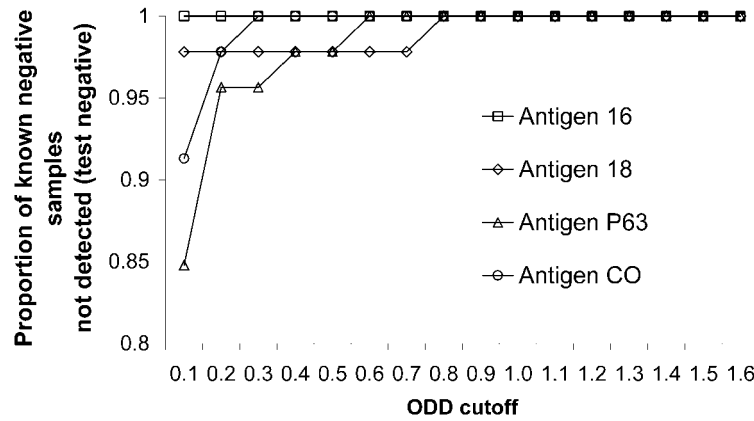


Figure 2. Proportion of known negative samples that tested negative for a given ODD cutoff for each assay.

tested positive using an ODD cutoff of 0.2. Known positive samples became negative at ODD cutoff values of 0.6, 0.5, 0.4, 0.2, and 0.4 for antigens 16, 18, P63, CO, and N, respectively (Fig. 1). All known negative samples tested negative at any ODD cutoff by the ELISA assay using antigen 16 (Fig. 2). The same set of negative samples tested negative at ODD cutoff values of 0.8, 0.6, and 0.3 for the assays using antigens 18, P63, and CO, respectively (Fig. 2).

Taking into consideration the proportion of correctly identified known positive and negative samples for a given cutoff, an ODD cutoff of 0.1 would maximize the performance of the assays using antigens 16 and 18. Using an ODD cutoff of 0.1, the assay with antigen 16 correctly detected 100% (46 of 46) of the known positive and negative samples and the assay with antigen 18 correctly detected 100% (46 of 46) of the known positive samples and 98% (45 of 46) of the known negative samples. For the assay using antigen P63, an ODD cutoff of 0.2 identified 100% (46 of 46)

of known positive samples and 96% (44 of 46) of known negative samples; an ODD cutoff of 0.1 for this assay would identify only 85% (39 of 46) of the known negative samples. The commonly used ODD cutoff of 0.2 for the assay with antigen CO yielded a correct result in 98% (45 of 46) of both known positive and negative samples.

An ODD cutoff corresponding to 3 SD from the mean of the known negative samples (Table 2) would correspond to cutoffs of 0.1, 0.11, 0.19, 0.2, and 0.13 for antigens 16, 18, P63, CO, and N, respectively. Assuming these ODD cutoffs for each assay, all known positive samples tested positive by each assay, except for antigen CO where 45 of the 46 samples tested positive (98%). The proportions of negative samples that tested negative using the same ODD cutoffs were 100% (46 of 46), 100% (46 of 46), 98% (45 of 46), and 94% (43 of 46) for antigens 16, 18, P63, and CO, respectively.

The largest effect of using S/P values was observed

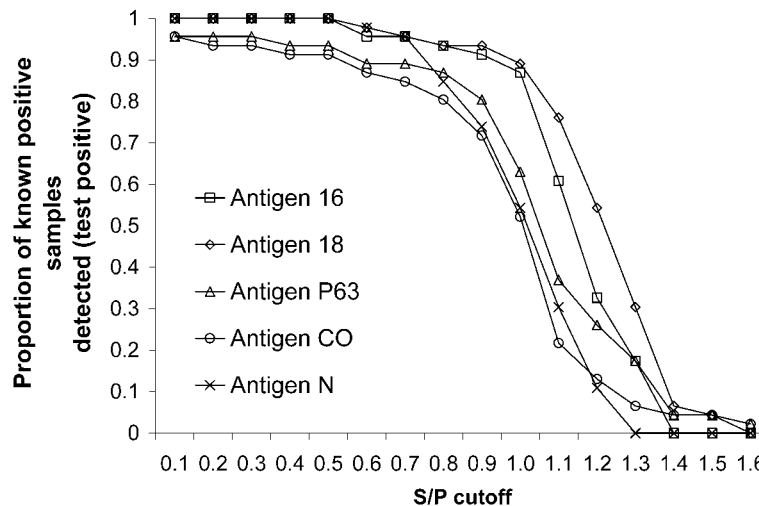
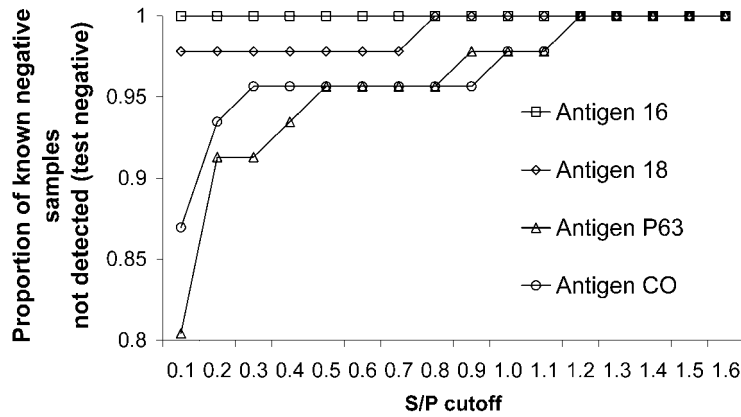


Figure 3. Proportion of known positive samples that tested positive for a given S/P cutoff for each assay.



**Figure 4.** Proportion of known negative samples that tested negative for a given S/P cutoff for each assay.

on assays with antigens P63, CO, and N. An S/P cutoff of 0.2 for the assay with antigen P63 corresponded to 96% (44 of 46) of the known positive samples with positive test results (Fig. 3) and only 91% (42 of 46) of the known negative samples with a negative test result (Fig. 4). For the assay using antigen CO, an optimal S/P cutoff of 0.3 detected 94% (43 of 46) of the known positive samples (Fig. 3) and 96% (44 of 46) of the known negative samples (Fig. 4). For the assay with antigen N, known positive samples started to test negative only above an S/P cutoff of 0.5 (Fig. 3).

**Evaluation on field samples.** The results of all 5 tests on 449 field samples using 3 different cutoff values are shown in Table 3. Results indicate that when a single cutoff ODD of 0.2 was used with all 5 antigens, the number of positive samples ranged from 241 to 387 (Table 3). There was less variation in the number of samples classified as positive when a cutoff value of mean of negative samples + 3 SD (356–387

samples) or the ROC optimal ODD cutoff (from 375 to 387) was used (Table 3).

**Evaluation of assay variability.** Calculation of CV from the ODD results of each of the 46 random samples, including person-to-person variation and day-to-day variation, showed an overall high variability regardless of the test. The test with the smallest overall CV was antigen 63, where 50% of the samples (median) had a CV of 32% or less (Fig. 5). The test with antigen N yielded the highest median CV, where 50% of the samples had a CV of 48% or less; however, some of the samples tested by antigen 18 showed either an extremely low (4%) or extremely high (>100%) CV (Fig. 5). The mean ODD values for each test for a given person and day are shown in Table 4. Analysis of these results showed that most of the variability for tests with antigen 16, 18, and P63 was explained by the different person performing the test ( $P < 0.01$  for all tests) but there was no significant difference between days 1 and 2 ( $P > 0.05$  for all tests) (Table 4). Conversely, for the test with antigen CO, there was a significant difference between days 1 and 2 but only for one of the persons performing the test ( $P = 0.0002$ ). No person-to-person or day-to-day statistically significant variation was observed in the test with antigen N. Reporting of results using S/P values showed a similar level of variability, where median CV ranged from 31% for the test with antigen P63 to 56% for antigen N. Similarly, S/P values showed significant variation at the person and day level for all 5 tests ( $P < 0.05$ ).

## Discussion

An indirect ELISA is being used as a routine diagnostic test for the detection of APV antibodies in Minnesota turkeys. When this ELISA was first standardized, no difference was found in the antigenicity of Colorado (APV/Colorado/turkey/97) and Minnesota isolates (APV/MN/turkey/1-a/97) of APV-C. On the

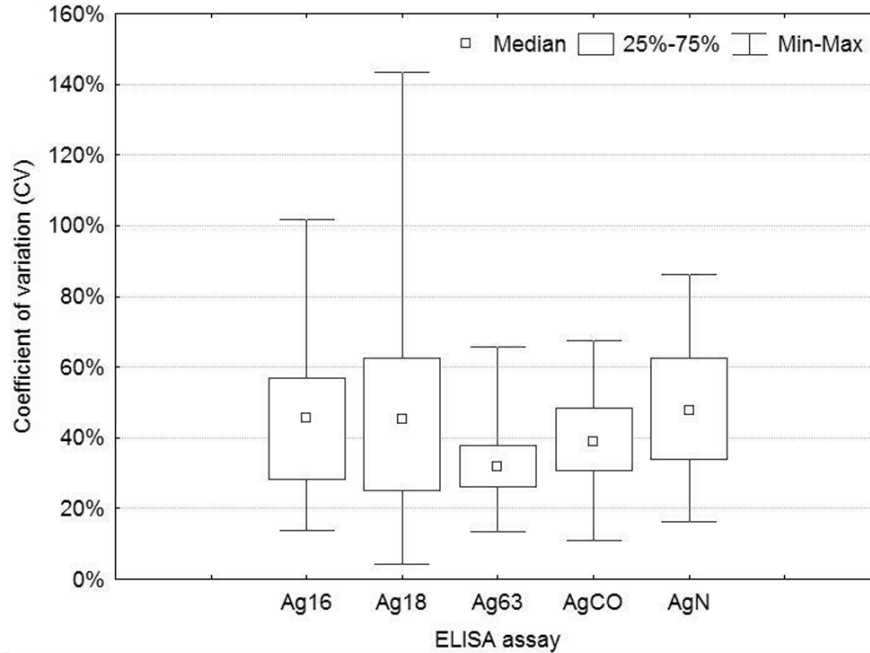
**Table 3.** Number of field samples that tested positive with 5 different antigens and various cutoffs ( $n = 449$ ).

Antigen name	ODD cutoff		
	ODD $\geq$ 0.2	Mean negative + 3 SD*	Optimal ODD†
MN-16	246 (55%)	366 (82%)	366 (82%)
MN-18	241 (54%)	356 (79%)	375 (84%)
MN-1a (P63)	370 (82%)	372 (83%)	370 (82%)
APV-CO	387 (86%)	387 (86%)	387 (86%)
N-protein	287 (64%)	375 (84%)	NT‡

\* Calculated as the mean of the known negative samples plus 3 SD, corresponding to ODD cutoffs of 0.1, 0.11, 0.19, 0.2, and 0.13 for antigens 16, 18, P63, CO, and N, respectively.

† Optical density difference cutoffs obtained from ROC plots, corresponding to cutoffs of 0.1 for antigens 16 and 18, and of 0.2 for antigens P63 and CO.

‡ Not tested.



**Figure 5.** Box-plot of the overall CV of ODD ELISA results for each of 5 different tests run on the same 46 samples by 2 people on 2 different days.

basis of these results, it was decided to routinely use the Colorado isolate as the antigen in the ELISA test.<sup>3</sup> The purpose of this study was to compare the antigenicity of older and newer isolates of APV-C to determine whether this can explain the recent ‘discrepant’ results obtained with the ELISA test based on the Colorado isolate. Differences in the results of ELISA due to antigenic variation have previously been suggested.<sup>7,24</sup> One of the 5 antigens used in this comparison was a vaccine strain of APV-C (P63).<sup>17</sup> The use of vaccine strains as antigens for the detection of antibodies has been suggested earlier.<sup>7,24</sup> An ELISA based on recombinant nucleoprotein was also used because the N-protein has 99.7% homogeneity among different isolates of APV.<sup>5</sup> Evaluation of the tests on field sam-

ples using a single ODD cutoff of 0.2 for all 5 tests resulted in a large difference in the number of samples classified as positive (241–387; Table 3), suggesting the potential for misclassification error. Between 356 and 387 samples were classified as positive by all 5 antigens (using a cutoff of mean negative ODD + 3 SD), indicating a lack of significant antigenic variation in APV-C isolates circulating in Minnesota between 1997 and 2000. The similar results found when the ROC-based cutoffs were used suggested the need for standardization according to the antigen being used rather than having a common cutoff point.

The accuracy and repeatability of a diagnostic test are essential for effective evaluation of results. In ELISA, many correction factors have been used to

**Table 4.** Mean ODD and S/P of 46 samples tested by 5 ELISA assays based on different antigens. The 5 tests were performed on the same samples by 2 different persons on 2 different days.

APV as antigen	Day	ODD		S/P	
		Person 1	Person 2	Person 1	Person 2
APV/MN/turkey/16/2000	1	0.32	0.26	0.57	0.45
APV/MN/turkey/16/2000	2	0.31	0.20	0.48	0.39
APV/MN/turkey/18/2000	1	0.35	0.29	0.44	0.34
APV/MN/turkey/18/2000	2	0.32	0.30	0.40	0.38
APV/MN/turkey/1-a/97	1	0.23	0.40	0.43	0.64
APV/MN/turkey/1-a/97	2	0.31	0.36	0.50	0.43
APV/Colorado/turkey/97	1	0.41	0.54	0.64	0.62
APV/Colorado/turkey/97	2	0.44	0.32	0.56	0.38
N-protein	1	0.28	0.32	0.57	0.47
N-protein	2	0.33	0.26	0.76	0.48

make the test repeatable, especially in single-dilution ELISA tests.<sup>22</sup> Currently, the subtraction of negative well OD from positive (antigen coated) well OD is able to reduce some of the background noise in APV ELISA. However, results from this study confirm the fact that personnel conducting the test may contribute substantially to some of the test variability, especially in low-positive cases with ODD near the cutoff point.

### Acknowledgements

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### Sources and manufacturers

- a. Sigma Chemical Co., St. Louis, MO.
- b. Kirkegaard and Perry Laboratories, Gaithersburg, MD.
- c. Immunolon 1B, Dynatech, Chantilly, VA.

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