(51) International Patent Classification*: A61B

(21) International Application Number: PCT/US01/41537

(22) International Filing Date: 2 August 2001 (02.08.2001)

(25) Filing Language: English

(30) Priority Data:
- 09/630,757 2 August 2000 (02.08.2000) US
- 60/280,719 2 April 2001 (02.04.2001) US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier applications:
- US 60/280,719 (CIP) Filed on 2 April 2001 (02.04.2001)
- US 09/630,757 (CIP) Filed on 2 August 2000 (02.08.2000)


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(81) Designated States (national): CA, US.

(84) Designated States (regional): European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR).

Published:
— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: AVIAN PNEUMOVIRUS DIAGNOSTIC SYSTEM

(57) Abstract: A method for detecting an avian pneumovirus (APV) infection is described that includes the use of an isolated APV polypeptide and/or anti-APV polypeptide antibodies. Antibodies directed to APV polypeptides as well as articles of manufacture that include isolated APV polypeptides and/or anti-APV polypeptide antibodies are also described.
AVIAN PNEUMOVIRUS DIAGNOSTIC SYSTEM

TECHNICAL FIELD

This invention relates to methods for detecting avian pneumovirus (APV) infections in birds, and more particularly to the use of isolated APV proteins such as the APV matrix (M) protein and/or the APV nucleocapsid (N) protein for detecting APV infections in birds.

BACKGROUND

Avian pneumovirus (APV) is a member of the Paramyxoviridae family of viruses. Pringle, Arch. Virol. 141:2251-6 (1996). It is the etiological agent of turkey rhinotracheitis, causing an acute upper respiratory tract infection characterized by coughing, nasal discharge, tracheal rales, foamy conjunctivitis and sinusitis in young poults. In laying birds, there is a transient drop in egg production along with mild respiratory tract illness. Jones, Avian Pathol., 25:639-48 (1996). While uncomplicated cases of APV infection usually result in low mortality, secondary bacterial and/or viral infections can result in up to 25% mortality. Id.

SUMMARY

The invention features methods for detecting APV infection. The methods include contacting an isolated APV polypeptide with an avian antibody source under conditions wherein the APV polypeptide binds to an anti-APV polypeptide antibody, if present in the avian antibody source, to form an antibody-polypeptide complex; and detecting the presence or absence of the antibody-polypeptide complex, wherein the presence of the antibody-polypeptide complex is indicative of or correlates with the occurrence of the APV infection. The APV infection can be from any APV isolate including the fourteen or so known APV isolates and in particular may be an APV isolate such as a European A, European B, Colorado, Minnesota-1a, Minnesota-1b, and a Minnesota-2a isolate.

Methods for detecting an APV infection also include providing a mixture that includes a presenting molecule and an APV polypeptide, contacting the mixture with an avian antibody source under conditions wherein the mixture binds to an anti-APV polypeptide antibody, if the anti-APV polypeptide antibody is present in the avian antibody source, to form an antibody-mixture complex; and detecting the presence or absence of the antibody-mixture complex. The presence of the antibody-mixture complex typically correlates with the occurrence of an APV infection. The presenting molecule can be a second anti-APV polypeptide antibody, and can be derived from an animal species other than the animal species from which the avian antibody source originates (e.g., a non-avian animal species). The APV polypeptide can be an isolated APV polypeptide (e.g., a recombinant APV polypeptide or an APV polypeptide isolated from Vero cells). This method is particularly useful for APV infections by an APV U.S. isolate such as a Colorado, a Minnesota-1a, a Minnesota-1b, or a Minnesota-2a isolate.

Methods of the invention further include contacting a presenting molecule with a biological sample under conditions wherein the presenting molecule binds to an APV polypeptide, if the APV polypeptide is present in the biological sample, to form a presenting molecule-APV polypeptide mixture, contacting the mixture with a mixture-recognizing molecule to form a complex; and detecting the presence or absence of the complex. Generally, the presence of the complex correlates with the occurrence of an APV infection.
In addition, methods of the invention include contacting an APV polypeptide with a first APV polypeptide-recognizing molecule and a second APV polypeptide-recognizing molecule to form a three-part complex, and detecting the presence or absence of said complex, wherein the presence of said complex correlates with the occurrence of an APV infection. The first and/or the second APV polypeptide-recognizing molecule can be anti-APV polypeptide antibodies. The anti-APV polypeptide antibody corresponding to the second APV polypeptide-recognizing molecule can be from avian antibody source. The APV polypeptide can be recombinant or can be from a biological sample.

An avian antibody source can be isolated from a bird, including domestic and wild birds, at least 10 days post-infection of the bird with APV. A biological sample can be isolated from a bird at least 4 days post-infection. Suitable domestic birds include chickens, turkeys, geese and ducks. Turkeys are particularly useful.

An isolated APV polypeptide can be encoded by an APV gene such as an N, P, M, F, M2, L, SH or G gene. Polypeptides encoded by an APV M gene or N gene are particularly useful, and can be a fragment of a full-length protein (e.g., a fragment including amino acids 172-201 of the M protein (SEQ ID NO:1) or a fragment including amino acids 78-91 of the N protein (SEQ ID NO:2)). The APV M gene or N gene can be from a European A, European B, Colorado, Minnesota-1a, Minnesota-1b, or Minnesota-2a isolate.

An isolated APV polypeptide, a presenting molecule, a mixture-recognizing molecule, a mixture, a complex, or immunoglobulins present in the avian antibody source can be immobilized on a solid substrate, such as a dipstick, microtiter plate, bead, affinity column, an immunoblot membrane, or other suitable solid substrate.

Detecting an antibody-polypeptide or other complex can include performing an enzyme-linked immunoassay including competitive ELISAs, radioimmunoassay, an immunoprecipitation, a fluorescence assay, or a particulate based assay. The detecting step can include contacting the antibody-polypeptide or other complex with an indicator molecule that selectively binds to an anti-APV polypeptide antibody, a mixture-recognizing molecule or to a complex, and detecting the presence or absence of the indicator molecule.
The invention also features an article of manufacture for detecting anti-APV polypeptide antibodies in an avian antibody source that includes an isolated APV polypeptide as described above. A presenting molecule also can be included in an article of manufacture of the invention. Further, a presenting molecule and an isolated APV polypeptide can be provided as a mixture in an article of manufacture. Two or more isolated APV polypeptides can be present in the article of manufacture, wherein different APV genes (e.g., N, P, M, F, M2, L, SH or G) encode the isolated polypeptides. Isolated APV polypeptides included in an article of manufacture of the invention can be encoded by genes from different APV isolates such as a European A, European B, Colorado, Minnesota-1a, Minnesota-1b, or Minnesota-2a isolate. Further, an isolated APV polypeptide, a presenting molecule or a polypeptide-presenting molecule mixture provided in an article of manufacture can be immobilized on a solid substrate.

An article of manufacture can include packaging material and an isolated APV polypeptide within the packaging material, wherein the packaging material contains a label indicating that the presenting molecule and/or the isolated APV polypeptide can be used for detecting the presence or absence of antigens or anti-APV polypeptide antibodies in birds. An article of manufacture further can include an indicator molecule, e.g., an indicator molecule that selectively binds to the anti-APV polypeptide antibodies produced by the bird species from which the avian antibody source is obtained. An article of manufacture can include reagents for detecting a plurality of avian infections including viral infections. The plurality of avian viral infections can be APV and at least one virus selected from the group consisting of avian encephalitis virus, avian influenza, avian leucosis, fowl pox, infectious bronchitis virus, infectious bursal disease virus, Newcastle disease virus, and reovirus.

The invention features an isolated APV polypeptide encoded by an APV N gene or an isolated APV polypeptide encoded by an APV M gene. Purified antibodies that selectively bind to an M polypeptide also are featured, as are purified antibodies that selectively bind to an N polypeptide.

Advantages of the invention include reliably detecting APV infection in individual birds and flocks of birds. Early and accurate detection can facilitate proper diagnosis and
treatment of infected birds and may reduce economic losses due to APV and/or other related secondary infections.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used to practice the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. Amino acid designations may include full name, three letter, or single letter designations as commonly understood by one of ordinary skill in the art to which this invention belongs. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

DESCRIPTION OF DRAWINGS

FIG. 1 is a graph of the presence of APV-specific IgG in sera of APV-infected turkeys detected by an APV protein ELISA. Two-fold serial dilutions of known positive turkey sera (n=10) were tested in plates coated with recombinant M protein. Pooled serum samples from flocks known to be negative for APV infection were used as a negative control. The results are plotted as mean plus or minus standard error.

FIG. 2 is a comparison of an APV protein ELISA with the routine ELISA for detection of APV-specific IgGs from experimentally infected turkeys. 34 turkeys were inoculated with live APV/MN-1a and serum was collected 4 weeks later. A 1:40 dilution of each sample was applied to wells of plates coated with either recombinant M protein or a cell lysate from an APV-infected cell. Detection was with an HRP-conjugated goat anti-turkey IgG secondary antibody. The A<sub>490</sub> values from the M protein-coated plates were consistently higher than those from the cell lysate-coated plates (p < 0.001).

FIG. 3 is a graph of the reactivity of hyperimmune sera from APV/MN-1a, European A and European B isolates using an N protein antibody ELISA. ELISA optical density (OD) values correspond to the difference between the mean absorbance (A<sub>490/405</sub>)
of each hyperimmune sera in wells coated with N protein-specific serum and wells coated with preimmune serum.

**DETAILED DESCRIPTION**

The present invention involves methods, reagents, and articles of manufacture (kits) useful for detecting APV infections in birds, including wild and domesticated birds such as sparrows, swallows, starlings, pheasants, geese, chickens, turkeys, and ducks. The methods described herein can be used for detecting infection by any APV isolate including, for example, the European A, European B, Colorado (CO), Minnesota-1a (MN-1a), Minnesota-1b (MN-1b), and Minnesota-2a (MN-2a) isolates. In particular, methods of the invention can detect infection by United States (U.S.) isolates such as the CO, MN-1a, MN-1b, or MN-2a isolates.

As APV elicits an immune response in avian species, anti-APV polypeptide antibodies are detectable in the sera of birds following APV infection. Thus, an APV infection can be detected by assessing whether anti-APV polypeptide antibodies are present or absent in biological samples obtained from birds. An APV infection also can be detected by assessing whether APV polypeptides are present or absent in biological samples obtained from birds. As used herein, “infection” is understood to include current infections (i.e., the virus is present and multiplying in the bird) and past infections in a bird that are sufficient to elicit a detectable immune response in the bird. “Anti-APV polypeptide antibody” refers to an antibody that has specific binding affinity for an APV polypeptide, such as an M polypeptide or an N polypeptide.

Biological samples can be referred to herein as “avian antibody sources” and such samples include, for example, whole blood, plasma, serum, feces, choanal swabs, nasal turbinate tissues or swabs, tracheal swabs, lung tissue and any other material from a bird from which antibodies or antigens can be detected or purified either directly or indirectly.

APV infection can be detected in avian antibody sources isolated from birds at various times after the birds are exposed to APV. For example, antibodies generally can be first detected in a bird at about 10 to about 14 days after viral infection, and typically can be detected for at least weeks, months, and oftentimes over the lifetime of a bird. Viral particles including APV polypeptides generally can be detected in a bird within
about 4 days post-viral infection, and can typically be detected for up to about 7 to about 14 days post-infection. By way of example, anti-APV polypeptide antibodies can be detected in an avian antibody source at least about 10 days, from about 10 days to about 10 weeks, at least about 15 days, at least about 15 weeks, and at least about 8 weeks post-infection. In addition, APV infection can be detected in a biological sample at various times after the birds are exposed to APV, including, for example, at least about 4 days, from about 4 days to about 10 days, at least about 15 days, and at least about 2 weeks post-infection.

APV infections can be detected by contacting an isolated APV polypeptide with an avian antibody source under conditions wherein the APV polypeptide can bind to an anti-APV polypeptide antibody in the avian antibody source, to form an antibody-polypeptide complex. The term “isolated polypeptide” refers to a polypeptide that has been removed from its natural environment, i.e., it has been separated from cellular and/or viral components that naturally accompany it. Typically, the polypeptide is isolated when it is at least 60% (e.g., 70%, 80%, 90%, 95%, or 99%), by weight, free from proteins and naturally occurring organic molecules that are naturally associated with it.

In addition, proteins, including immunoglobulins, and other compounds capable of adhering or binding to APV polypeptides can be used to present an APV polypeptide for subsequent detection. For example, a presenting molecule such as a protein, an immunoglobulin, or other similar compound can be immobilized on a solid substrate. The presenting molecule can be contacted with an APV polypeptide to form a mixture or combination. The APV polypeptide can be an isolated APV polypeptide (for example, a recombinant APV polypeptide) or an APV polypeptide contained within a biological sample such as a choanal or turbinate swab. The resulting presenting molecule-APV polypeptide mixture can be contacted with an anti-APV polypeptide antibody or other suitable molecule capable of recognizing and adhering to an APV polypeptide or a presenting molecule-APV polypeptide mixture. The resulting complex can be detected using known methods. It is to be understood that the mixture can be immobilized on a solid substrate or, alternatively, the method steps can be performed without immobilizing either the presenting molecule or the mixture. Such methods are particularly useful for
presenting an N APV polypeptide and detecting the presence or absence of an anti-APV polypeptide antibody in an avian antibody source, or for directly detecting the presence or absence of an N APV polypeptide in an avian biological sample.

The APV genome is a linear, single-stranded RNA molecule of approximately 13.3 kilobases (kb) that encodes 8 polypeptides (N, P, M, F, M2, SH, G, and L). See, *e.g.*, Randhawa et al., *J. Virol.*, 71:9849-54 (1997) and references therein. Any one of the eight polypeptides encoded by the APV genome can be used in the methods of the invention. Furthermore, multiple isolated APV polypeptides may be used in combination to detect infection by APV and/or to distinguish between different APV isolates. An "APV polypeptide" is a polypeptide that is encoded by a complete or partial gene present in the genome of an APV and includes any chain of at least five amino acids that contains an epitope recognized by an anti-APV polypeptide antibody. Thus, isolated APV polypeptides can be full-length proteins or fragments of a full-length protein. For example, an isolated surface glycoprotein (G) polypeptide, fusion (F) polypeptide, matrix (M) polypeptide, or nucleocapsid (N) polypeptide, can be used. As described herein, the M protein is antigenic and antibodies to M protein can be observed consistently among field-collected sera from infected birds. The N protein also is antigenic and antibodies to N protein can be observed consistently among field-collected sera from infected birds. Thus, M polypeptides and N polypeptides are particularly useful.

G polypeptides have 99% identity among viruses of the same group and 38% similarity between APV subtypes. See, Seal et al., *Virus Res.*, 66:139-47 (2000). F polypeptides from U.S. isolates contain at least nine conserved amino acid substitutions, and share 72% and 71% amino acid sequence identity with European A and B isolates, respectively. The M gene has 98% nucleotide similarity among the Colorado and Minnesota isolates and 73% sequence similarity between European A and B isolates. See, Seal, *Virus Res.*, 58:45-52 (1998). The N protein exhibits 100% sequence identity between the European A and B isolates, and 70% identity between the U.S. Colorado isolate and either the European A or B isolate.

Fragments of APV polypeptides that have amino acid sequences conserved among multiple isolates are also useful for detecting APV. For example, amino acids 172 to 201, *i.e.*, SESATVEAAISGEADQAITQARIAPYG (SEQ ID NO:1) (as described in Seal
(1998), supra), of the M polypeptide are highly conserved among the Colorado, European A and European B isolates, and amino acids 78-91, i.e., RTQILKNSGSEVQ (SEQ ID NO:2) (see, for example, GenBank Accession Nos. AF176590, APU39295, and APU39296), of the N polypeptide are highly conserved among the Colorado, European A and European B isolates. Fragments containing these sequences can be used to bind anti-APV polypeptide antibodies. It is to be understood, however, that other APV polypeptide fragments, including fragments shorter than amino acids 172 to 201 of an APV M protein or amino acids 78-91 of an APV N protein, may be used. Accordingly, fragments as small as a single epitope may be useful to detect APV infections. Alternatively, variable regions of an APV polypeptide can be used to distinguish between different APV isolates, e.g., amino acids 120 to 140 of the M polypeptide (see Seal (1998), supra) or amino acids 156-185 of the N polypeptide contain divergent amino acids among the Colorado and European A and B isolates. Accordingly, polypeptide fragments that do or do not elicit cross-reactivity between various APV isolates can be selected.

Detecting APV Infection

Isolated APV polypeptides can be immobilized on a solid substrate, such as a 96-well plate, using known methodologies (e.g., contacting a microtiter plate with an APV polypeptide in the presence of sodium carbonate buffer (pH 9.5) at 37°C from about at least 10 min to about 10 hrs (e.g., from about 1 to about 2.5 hrs). The immobilized APV polypeptide then can be contacted with an avian antibody source under conditions such that anti-APV polypeptide antibodies, if present in the avian antibody source, can bind to the polypeptide to form an antibody-polypeptide complex. Suitable conditions for generating an antibody-polypeptide complex include incubation in an appropriate buffer (e.g., phosphate-buffered saline (PBS), pH 7.2) at room temperature from about at least 10 min to about 10 hrs (e.g., from about 1 to about 2.5 hrs). Thereafter, unbound material can be washed away and an antibody-polypeptide complex detected. Such an assay is sometimes referred to herein as an APV protein immunoassay (e.g., an APV protein ELISA, e.g., an M or N protein ELISA).

Conversely, proteins present in the avian antibody source, including immunoglobulins, can be immobilized on the solid substrate, e.g., by drying the avian
antibody source on the solid substrate or by interaction of the immunoglobulins in the avian antibody source with anti-IgG antibodies immobilized on the solid substrate. The immobilized immunoglobulins then can be contacted with an isolated APV polypeptide under conditions such as those described above that are sufficient to form an antibody-polypeptide complex. Methods for immobilizing proteins such as immunoglobulins on solid substrates are known.

A presenting molecule such as an anti-APV polypeptide antibody can be immobilized on a solid substrate using known methodologies and contacted with an APV polypeptide under suitable conditions for producing a presenting molecule-APV polypeptide mixture. Excess APV polypeptide can be removed by washing, and the immobilized mixture contacted with an avian antibody source (containing anti-APV polypeptide antibodies if an APV infection is present) or other mixture-recognizing molecule under suitable conditions to produce a complex. Complexes can be subsequently detected by known methods using, for example, an indicator molecule. An assay in which a mixture is contacted with an avian antibody source is sometimes referred to herein as an APV protein antibody immunoassay (e.g., an N polypeptide antibody ELISA). Alternatively, an assay in which the APV polypeptide of a mixture is from an avian biological sample is sometimes referred to herein as an APV protein antigen immunoassay (e.g., an N polypeptide antigen ELISA).

Detecting the presence of complexes formed using the methods of the invention (e.g., an antibody-polypeptide complex or an antibody-mixture complex) is indicative of an APV infection. Assay formats for detecting such complexes can include enzyme-linked immunoassays (ELISA) such as competitive ELISAs, radioimmunoassays (RIAs), fluorescence assays, chemiluminescent assays, immunoblot assays (Western blots), particulate-based assays, and other known techniques. Typically, isolated APV polypeptides or anti-APV polypeptide antibodies can be immobilized on solid substrates such as dipsticks, microtiter plates, particles (e.g., beads), affinity columns, and immunoblot membranes. See U.S. Patent Nos. 5,143,825, 5,374,530, 4,908,305, and 5,498,551 for exemplary descriptions of solid substrates and methods for their use. In some embodiments, complexes are formed in solution. Such complexes can be detected

In general, complexes can be detected using an indicator molecule having specific binding affinity for the complex. As used herein, an "indicator molecule" is any molecule that allows the presence of a given polypeptide or antibody, or complexes to be visualized, either with the naked eye or with an appropriate instrument. Typically, the indicator molecule is an antibody that selectively binds antibodies from the bird species from which the avian antibody source or anti-APV polypeptide antibodies were obtained, e.g., an anti-turkey IgG antibody (Rockland Immunochemicals, Gilbertsville, PA).

Indicator molecules can be detected either directly or indirectly by standard methodologies. See, e.g., *Current Protocols in Immunology*, Ch. 2 and 8, Coligan et al., (eds.), John Wiley & Sons (1996). For direct detection, the indicator molecule or the APV polypeptide (when immunoglobulins are immobilized on a solid substrate) can be labeled with a radioisotope, fluorochrome, other non-radioactive label, or any other suitable chromophore. For indirect detection methods, enzymes such as horseradish peroxidase (HRP) and alkaline phosphatase (AP) can be attached to the indicator molecule, and the presence of the antibody-polypeptide or antibody-polypeptide-antibody complex can be detected using standard assays for HRP or AP. Alternatively, the indicator molecule can be attached to avidin or streptavidin, and the presence of the complex can be detected with biotin conjugated to, for example, a fluorochrome, or vice versa.

*Detecting Infections in Birds*

The methods described herein can be used to detect multiple isolates of APV using a single APV polypeptide. For example, recombinant M polypeptides from the Colorado strain of APV (APV/CO) and from a Minnesota isolate (APV/MN-2a) were equally effective for detecting APV isolates, including European A and United States isolates. Cross-reactivity was detected to the infectious bronchitis virus. Use of the M polypeptide was highly sensitive as 97.1% of experimentally infected turkeys were detected, compared to only 54.5% of turkeys detected when using intact APV as the antigen. All samples that were determined to be positive using the M polypeptide also
were positive by Western analysis and all negative samples were negative by Western analysis. In contrast, 12 samples that were deemed positive when using intact APV particles as the antigen were negative in Western blot analysis. Moreover, the methods described herein using isolated APV polypeptides to detect anti-APV polypeptide antibodies are at least six times more sensitive than isolating virus particles from field cases showing overt APV disease.

The methods described herein can be used to detect multiple isolates of APV using anti-APV polypeptide antibodies. For example, anti-N protein antibodies generated towards a recombinant N protein from APV/CO were highly effective for detecting APV/US isolates, including Colorado and Minnesota (MN-1a) isolates. Using a presenting molecule (e.g., rabbit anti-APV polypeptide antibodies) bound to a recombinant N protein to detect anti-N polypeptide antibodies in a bird was 100% sensitive (all experimentally-infected turkeys were scored as positive) and 100% specific (all viral-free turkeys were scored as negative). In addition, such an assay detected APV infection in serum samples from 37 birds that routine ELISA did not detect, and 2 samples that were negative using a presenting molecule bound to a recombinant N protein assay were positive by routine ELISA. These two samples were confirmed to be negative based on Western analysis using recombinant N protein or purified APV proteins. Thus, an immunoassay format such as an N polypeptide antibody ELISA is more sensitive and specific than routine ELISA.

Using a presenting molecule to detect an APV polypeptide in a biological sample also was highly sensitive and specific in detecting infected turkeys, compared to the number of infected turkeys detected when using a routine ELISA. Thus, an immunoassay format such as an N polypeptide antigen ELISA is more sensitive and specific than routine ELISA.

The methods of the invention are useful for determining the nature of an infection affecting a flock. Thus, the methods described herein can be used to determine if a bird has an APV infection, a bacterial infection, a mycoplasma infection such as a *Mycoplasma gallisepticum* or *Mycoplasma synoviae* infection, or another viral infection, such as infection caused by avian encephalitis virus (AEV), avian influenza virus, avian leukosis virus (ALV), fowl pox, infectious bronchitis virus (IBV), infection bursal disease
virus (IBD), Newcastle disease virus (NDV, also known as paramyxovirus-1, PMV-1), PMV-2, PMV-3, or a reovirus. To do so, the isolated APV polypeptide or anti-APV polypeptide antibodies can be used in conjunction with reagents for detecting other viruses and/or other avian related diseases, such as isolated polypeptides, inactivated viral particles, bacteria, other infectious agents, or antibodies. For example, an isolated APV polypeptide or anti-APV polypeptide antibody can be immobilized on a solid substrate and then other suitable polypeptides or antibodies capable of detecting different avian diseases can be immobilized in different, discrete regions, of the solid substrate. Appropriate immunoassays can be completed using a biological sample as described above.

*Production of Isolated APV Polypeptides*

Isolated polypeptides that are useful for detecting an APV infection can be obtained from any source, including recombinant DNA technology, chemical synthesis, and purification from Vero cells or a natural source. Methods for producing and isolating APV polypeptides are known and include the following illustrative examples. Recombinant APV polypeptides can be produced by ligating a nucleic acid sequence encoding the polypeptide into a nucleic acid construct such as an expression vector, and transforming a bacterial or eukaryotic host cell with the expression vector. See, for example, GenBank Accession No. AF072682 for a sequence of a suitable M polypeptide or GenBank Accession No. AF176590 for a sequence of a suitable N polypeptide. In general, nucleic acid constructs include a regulatory sequence operably linked to a nucleic acid sequence encoding an APV polypeptide. Regulatory sequences do not typically encode a gene product, but instead affect the expression of the nucleic acid sequence. As used herein, "operably linked" refers to connection of the regulatory sequences to the nucleic acid sequence in such a way as to permit expression of the nucleic acid sequence. Regulatory elements can include, for example, promoter sequences, enhancer sequences, response elements, or inducible elements.

In bacterial systems, a strain of *E. coli* such as M15 can be used. Suitable *E. coli* vectors include, without limitation, the pGEX series of vectors that produce fusion proteins with glutathione S-transferase (GST). Transformed *E. coli* are typically grown
until they reach an exponential phase and then the cells are stimulated with isopropyl-\text{-}\text{thiogalactopyranoside} (\text{IPTG}) before harvesting. In general, such fusion proteins are soluble and can be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites such that the cloned target gene product can be released from the GST moiety.

The prokaryotic expression vector pQE-30 (QIAGEN Inc., Valencia, CA) also can be used. Polypeptides that are expressed from pQE-30 have 9 amino acids (the RGS-6XHis tag) added to their N-terminus, allowing the polypeptides to be purified using antibodies to either the RGS or 6XHis tag, or using columns that bind to the 6XHis moiety, such as a nickel nitriloacetate column. Alternatively, the nucleic acid encoding an APV polypeptide can be modified such than an epitope for a commercially available monoclonal antibody is expressed at the N- or C-terminus of the polypeptide. For example, there are several epitopes of the human c-myc protein for which corresponding monoclonal antibodies are commercially available that have been successfully used for purifying proteins. Evan et al. (\textit{Mol. Cell. Biol.}, 5: 3610-16 (1985)), describes monoclonal antibodies that bind to epitopes of the human c-myc protein, at least one of which is available from BAboCO (Richmond, CA) and the American Type Culture Collection (ATCC; CRL-1729). See also, Kolodziej and Young, \textit{Methods Enzymol.}, 194:508-19 (1991).

In eukaryotic host cells, a number of viral-based expression systems can be utilized to express polypeptides. A nucleic acid encoding an polypeptide can be cloned into, for example, a baculoviral vector such as pBlueBac (Invitrogen; Carlsbad, CA) and then used to co-transfect insect cells such as \textit{Spodoptera frugiperda} (Sf9) cells with wild-type DNA from \textit{Autographa californica} multiply enveloped nuclear polyhedrosis virus (AcMNPV). Recombinant viruses producing APV polypeptides can be identified by standard methodology. Alternatively, a nucleic acid encoding an APV polypeptide can be introduced into an SV40-, retroviral-, or vaccinia-based viral vector and used to infect suitable host cells.

Mammalian cell lines that stably express polypeptides can be produced by using expression vectors with appropriate control elements and a selectable marker. For
example, the eukaryotic expression vector pCDNA.3.1+ (Invitrogen) is suitable for expressing modified polypeptides in, for example, COS cells, HEK293 cells, or baby hamster kidney cells. After introducing the expression vector by electroporation, DEAE dextran, calcium phosphate, liposome-mediated transfection, or other suitable method, stable cell lines can be selected. Alternatively, transiently transfected cell lines are used to produce polypeptides. Polypeptides also can be transcribed and translated in vitro using wheat germ extract or rabbit reticulocyte lysate.

Polypeptides can be purified from conditioned cell medium by applying the medium to an affinity column (e.g., a Ni-NTA or GST column as described above for 6xHis-tagged or GST-fusion polypeptides, respectively). In addition, polypeptides can be purified from cell medium using an immunoaffinity column. For example, an antibody having specific binding affinity for an APV protein can be immobilized in a suitable column media and used to purify APV proteins. Further, polypeptides can be gel-purified (i.e., polypeptides can be separated by electrophoresis and the region of the gel corresponding to the predicted size of the desired polypeptide can be physically removed and the contents extracted).


APV polypeptides also can be isolated from intact APV viral particles using known methods. Intact viral particles can be isolated from blood of an APV infected bird or from infected cells in culture, e.g., Vero cells, and the viral proteins separated by known methodologies.

*Production of Anti-APV Polypeptide Antibodies*

The invention also features antibodies and fragments thereof having specific binding affinity for any isolated APV polypeptide, e.g., an M polypeptide or an N polypeptide. Antibodies having specific binding affinities for an APV polypeptide can be produced using standard methods. "Antibody" or "antibodies" includes intact molecules as well as fragments thereof that are capable of binding to an epitope of an APV polypeptide. Thus, the terms "antibody" and "antibodies" include polyclonal antibodies,
monoclonal antibodies, humanized or chimeric antibodies, single chain Fv antibody fragments, Fab fragments, and F(ab)_2 fragments. Monoclonal antibodies are particularly useful. The term "epitope" refers to an antigenic determinant on an antigen to which the paratope of an antibody binds. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains, and typically have specific three-dimensional structural characteristics, as well as specific charge characteristics. Epitopes generally have at least five contiguous amino acids.

In general, an M polypeptide, N polypeptide, or other isolated APV polypeptide or fragment thereof is produced as described above, i.e., recombinantly, by chemical synthesis, or by purification of the native protein, and then used to immunize animals. Various host animals including, for example, rabbits, chickens, mice, guinea pigs, and rats, can be immunized by injection of the protein of interest. Depending on the host species, adjuvants can be used to increase the immunological response. Adjuvants include Freund's adjuvant (complete and/or incomplete), mineral gels such as aluminum hydroxide, surface-active substances such as lysolecthin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin (KLH), and dinitrophenol.

Polyclonal antibodies are heterogeneous populations of antibody molecules that are specific for a particular antigen, which are contained in the sera of the immunized animals. Monoclonal antibodies, which are homogeneous populations of antibodies to a particular epitope contained within an antigen, can be prepared using standard hybridoma technology. In particular, monoclonal antibodies can be obtained by any technique that provides for the production of antibody molecules by continuous cell lines in culture such as described by Kohler et al., Nature, 256:495 (1975), the human B-cell hybridoma technique of Kosbor et al., Immunology Today, 4:72 (1983) and/or Cole et al., Proc. Natl. Acad. Sci. USA, 80:2026 (1983), and the EBV-hybridoma technique of Cole et al., In Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc. pp. 77-96 (1983). Such antibodies can be of any immunoglobulin class including IgM, IgG, IgE, IgA, IgD, and any subclass thereof. The hybridoma producing the monoclonal antibodies of the invention can be cultivated in vitro or in vivo.

A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine
monoclonal antibody and a constant region derived from a human immunoglobulin.
Chimeric antibodies can be produced through standard techniques.

Antibody fragments having specific binding affinity for APV polypeptides can be
generated by known techniques. For example, such fragments include, but are not limited
to, F(ab)_2 fragments that can be produced by pepsin digestion of an antibody molecule,
and Fab fragments that can be generated by reducing the disulfide bridges of F(ab')_2
fragments. Alternatively, Fab expression libraries can be constructed. See, for example,
Huse et al., Science, 246:1275 (1989). Single chain Fv antibody fragments are formed by
linking the heavy and light chain fragments of the Fv region via an amino acid bridge
(e.g., 15 to 18 amino acids), resulting in a single chain polypeptide. Single chain Fv
antibody fragments can be produced through standard techniques. See, for example, U.S.
Patent 4,946,778.

Once produced, antibodies or fragments thereof are tested for recognition of an
APV polypeptide by standard immunoassay methods including, for example, ELISA or
RIA. See Short Protocols in Molecular Biology, Ch. 11, Ausubel et al., (eds.), Green
have equal binding affinities for recombinant and native proteins.

Article of Manufacture for Detecting APV Infections

Isolated APV polypeptides that are effective for detecting the presence of APV
infection in birds as described herein can be combined with packaging material and sold
as a kit for detecting APV infections. The articles of manufacture may combine one or
more isolated APV polypeptides, such as the M polypeptide, the N polypeptide, or
fragments thereof as described herein. Alternatively or additionally, a kit for detecting
APV infections can include presenting molecules, anti-APV polypeptide antibodies (e.g.,
monoclonal or polyclonal antibodies), and/or other useful molecules for detecting APV
infections in birds. Such articles of manufacture can include APV polypeptides and/or
anti-APV polypeptide antibodies immobilized on a solid support. Further, an article of
manufacture for detecting APV infections can contain one or more indicator molecules to
detect the formation of complexes. Components and methods for producing articles of
manufactures are well known in the art.
Articles of manufacture provided by the invention may further include polypeptides, antibodies, indicator molecules, and/or useful agents for detecting other avian diseases. Instructions describing how the various peptides and diagnostic molecules are effective for detecting the presence of APV or other infections in birds may be included in such kits.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

Example 1—Materials and Methods

A. Producing and Purifying APV M Protein

cDNA clones of an M gene (GenBank Accession No. AF072682) were isolated from both the Minnesota 2A (APV/MN-2a, GenBank Accession No. AF187154) and the Colorado (APV/CO, GenBank Accession No. AF072682) isolates of APV from the pCR-XL-TOPO vector (Invitrogen) by PCR. The TOPO vector containing APV isolate clones were obtained from Bruce Seal and were the same clones described in Seal et al., *Virus Research*, 66:139-47 (2000). The 5’ PCR primer had the sequence 5’-CGCGGATCCATGGAGTCTATCTTAG-3’ (SEQ ID NO:2) and was designed to anneal to the first 13 nucleotides of the M gene coding sequences (the initiator ATG codon is bolded). The sequence of the primer included a *BamHI* restriction site just 5’ to the M gene initiator codon (underlined). The 3’ primer had the sequence 5’-CGCGTGCACCTAAATAATATCAAGC-3’ (SEQ ID NO:3), and was designed to anneal to bases 834-849 of the M gene coding sequence (the complement of the TAG stop codon is bolded), and included a *SalI* restriction site just 3’ of the M gene sequence (underlined above). In the PCR product, the *SalI* site is located 3’ of the TAG stop codon. The resulting PCR reaction was a complete M coding sequence (initiator codon to stop codon) flanked by *BamHI* (5’) and *SalI* (3’) restriction sites.

PCR reactions were performed by denaturing for 4 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min, and a final extension at 72°C for 1 min. The reactions included 1 µl
genomic APV DNA, 100 pmol of each primer, and 10 nmol of each dNTP in a final
reaction volume of 50 µl (1 µl DNA; 5 µl 10X PCR buffer; 2.5 µl 20X MgCl₂; 1 µl 10
mM dNTP’s; 2 µl 3’ Primer (50 pmol/µl); 36 µl ddH₂O; 0.5 µl Taq polymerase).

PCR products were digested with BamHI and SalI. The two M-derived cDNAs
were subcloned into the BamHI and SalI sites of the pQE-30 vector (Qiagen Inc.,
according to the manufacturer’s specification) resulting in the M-derived cDNA being
operably linked to the regulatory sequences of the pQE-30 Vector. Both strands of the
cDNA clones in these expression vectors were sequenced using an automated sequencer.
All clones contained sequences corresponding to an APV M gene (GenBank Accession
No. AF072682 or AF187154). The pQE-30 vector has a sequence encoding arginine-
glycine-serine followed by six histidine amino acids (the RGS–histidine tag) just 5’ to the
BamHI site located in the vector’s cloning region. Proteins expressed using the pQE-30
vector were tagged at their N-termini with RGS–histidine.

Recombinant pQE-30 plasmids containing appropriate M cDNAs were introduced
into E. coli strain M15, which contains the repressor plasmid pREP4. Plasmid pREP4
constitutively expresses the lac repressor protein encoded by the lacI gene. M protein
expression was induced using 0.3 mM with IPTG for four hrs.

Expressed M proteins were purified using nickel-nitrilotriacetic acid (Ni-NTA)
metal-affinity chromatography matrices that bind to the RGS–histidine tag present in the
expressed proteins, under either native or denaturing conditions. Briefly, the E. coli cells
were pelleted by centrifugation in a phosphate wash buffer (50 mM NaH₂PO₄ (pH 8.0),
300 mM NaCl, 25 mM imidazole) containing 1 mg/ml lysozyme and followed by
sonication. Cellular debris was removed by centrifugation (30 min, 14,000 rpm in a
Beckman JA-20). The lysate was incubated with Ni-NTA beads for 1 hr at 4°C. The
bound column was washed with a phosphate wash buffer containing 25 mM imidazole
and the M proteins eluted using a phosphate buffer having 250 mM imidazole. Proteins
were also isolated under denaturing conditions by suspending the cell pellet in denaturing
lysis buffer, e.g., 8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris-HCl (pH 8.0) followed by
sonication. Triton-X 100 (0.1%) can be added to the sonicated suspension before
pelleting the cellular debris as described above. The lysate was then incubated with Ni-
NTA beads for 1 hr at room temperature, washed with the denaturing lysis buffer
(adjusted to pH 6.3) and then eluted. The purified proteins were analyzed by sodium
dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and confirmed by
Western blot analysis using antibodies against the RGS – histidine tag. To eliminate
contaminating E. coli proteins eluted from the nickel-nitrilotriacetic acid matrices, the M
proteins were concentrated using Centricon-10 concentrators, gel-purified by
electroelution, and dialyzed versus PBS.

B. Purification of APV Viral Particles from Cell Culture

APV/MN-1a- or APV/MN-2a-infected Vero cells (ATCC-CCL81) were harvested
72 hrs post-infection, lysed with 0.5% IGEPAL CA-630 and clarified at 10,000 xg for 20
min at 4°C. The APV was pelleted through a 30% (w/w) sucrose cushion at 25,000 rpm
for 5 hrs in an SW28 rotor. The pellet was resuspended in 10 mM Tris-HCl (pH 7.4), and
overlaid on a CsCl density gradient (density between 1.2 and 1.6), and centrifuged in an
SW41 rotor at 25,000 rpm overnight at 4°C. An opalescent band at a specific density
between 1.31 and 1.37 was collected and dialyzed in PBS. The APV preparation that had
passed through the sucrose gradient, but not through the CsCl gradient, was used as
partially purified virus.

C. Western Blot Analysis

Purified APV proteins or recombinant APV M proteins were separated using 15%
SDS-PAGE under reducing conditions and transferred to a polyvinylidene difluoride
(PVDF) membrane by electroblotting. The membrane was blocked for 1 hr with 10%
non-fat dry milk at room temperature before incubating with a 1:40 dilution of turkey sera
for 1 hr. The turkey sera were obtained from experimentally infected and field-infected
turkeys. The membrane was washed 3 times with Tris-borate buffer containing 0.05%
Tween-20, incubated with HRP conjugated-goat anti-turkey IgG (heavy and light chains)
(Kirkgaard & Perry Laboratories (KPL), Gaithersburg, MD), and detected by
chemiluminescence using high performance films (Catalog No. RPN2106, Amersham

D. Virus Isolation from Avian Sources

Nasal turbinate tissues or swabs from exposed asymptomatic birds (in barns where
other birds were showing overt clinical signs of APV), or from birds that had early signs
of infection (rales, mild turbinate swellings) were processed for APV isolation. The
samples were homogenized and cultured in chick embryo fibroblasts for 5 blind passages, then passaged in Vero cells. Cytopathic changes were observed in Vero cells as early as the second passage. In the absence of detectable cytopathic changes, passaging was continued for 5 times and RT-PCR was performed. An isolate was confirmed by immunohistochemistry using a non-avian (i.e., rabbit) polyclonal anti-APV polypeptide antibody.

E. Serum Specimens

Turkey sera were submitted to the Minnesota Veterinary Diagnostic laboratory (333 Gortner Avenue, St. Paul, MN 55108) for the detection of antibodies directed towards APV. A total of 184 turkey sera from different farms that reported clinical signs of APV disease in 1999 were used in this study. In addition, 34 sera from experimentally infected turkeys 28 days post-infection were analyzed for antibodies directed towards APV using an APV protein ELISA. Forty-one serum samples from sham-inoculated controls were also analyzed. Experimental turkeys were obtained from areas of Wisconsin that were free from APV disease.

F. Routine APV ELISA

A routine APV ELISA was performed as described (Chiang et al., J. Vet. Diagn. Invest., 12:381-4 (2000)) to compare ELISA results based on APV/CO or two Minnesota isolates, APV/MN-1a and APV/MN-2a. It was determined that the APV/US isolates gave similar results in ELISA. Therefore, the routine ELISA test was optimized using the APV/CO isolate. Briefly, Vero cells infected with APV/CO were lysed in 0.01 M PBS with 0.5% Nonidet P-40 and the lysate was clarified by centrifugation at 3000 xg for 10 min. Clarified cell lysates were used as positive antigen, while cell lysates from mock-infected Vero cells were used as negative control antigens. Alternate rows of an ELISA plate (Immuno1B, Dynatech, Chantilly, VA) were coated overnight at 4°C with a 1:320 dilution of the infected cell lysate and non-infected Vero cells in 0.05 M carbonate buffer, pH 9.6. Test sera diluted 1:40 and anti-turkey HRP conjugate (1:1500) diluted in ELISA blocking solution (KPL) were each incubated for 1 h at room temperature in 50 μl volume. The substrate chromogen solution consisted of 0.04% (w/v) ortho-phenylene diamine in 0.05 M citrate-phosphate buffer (pH 5.0) and freshly added 0.04% (v/v) H2O2. The results were expressed as the difference between the A490 reading of antigen-coated
wells and that of control lysate-coated wells of each serum. A sample with $A_{490}$ value of more than 0.2 was considered positive. It is to be understood that wavelengths other than 490 nm may be useful for making these measurements.

G. M Protein ELISA

Purified recombinant M protein was used as a positive antigen. Bovine serum albumin or recombinant Theiler’s murine encephalomyelitis virus 2C protein generated in the same E. coli system as the APV M protein were used as negative control antigens. The optimum concentration of M protein to coat the ELISA plate was chosen in such a manner that maximum binding could be obtained as determined in a checkerboard titration according to known methods. The plates were coated overnight at 4°C with 125 ng of M protein per well in 100 µl of 0.05 M carbonate buffer, pH 9.6. All other steps were identical to those of the routine APV ELISA as described in Chiang et al. (2000), supra.

H. Production of Recombinant M Protein

M protein cDNAs from the APV/CO or APV/MN-2a (from the ATG start sequence at position 14 to position 860) isolates was expressed in an E. coli system yielding APV M proteins of 282 amino acids. Each expressed M protein gave a single band of approximately 30 kilodaltons (kD) when analyzed by SDS-PAGE. The expressed M proteins were purified by 6x His-tag-based nickel nitriloacetic column according to the manufacturer’s instructions (Qiagen, Inc.). Further purification was accomplished by running the column-purified proteins on a polyacrylamide gel followed by electroelution in buffer containing 25 mM Tris base, 0.19 M glycine and 0.1% SDS. The purity of the electroeluted proteins was confirmed by SDS-PAGE followed by either Coomassie staining the gel or transferring the proteins to a PDVF membrane and performing Western blot analysis using anti-His-tag and/or anti-APV antibodies.

Example 2—Antigenicity of the M Protein in APV Infection

To determine if the M protein acts as an antigen during an immune response to APV infection in birds, recombinant M protein and partially purified APV virions were separated by SDS-PAGE and transferred to PDVF membranes. These membranes were then subjected to Western blot analysis using turkey sera from APV-infected flocks.
Turkey sera (1:40 dilution) were incubated with membranes, followed by a labeled secondary antibody (horseradish peroxidase-labeled goat anti-turkey IgG at a 1:20,000 dilution) that allowed chemiluminescent detection of proteins for which antibodies with specific binding affinities were present in the turkey sera. Positive sera reacted with several proteins in lanes containing partially purified APV virions, including a protein of approximately 30 kD that was similar in size to a protein detected in those lanes containing only recombinant M protein. Negative sera at the same dilution failed to detect any proteins in lanes containing either recombinant M protein or partially purified APV virions.

Sera were also tested from turkeys experimentally infected with APV/MN-2a. These sera reacted with recombinant M proteins expressed by cDNAs cloned from either APV/MN-2a or APV/CO, indicating that recombinant M proteins from these isolates share antigenic homology. Using partially purified APV, two proteins of approximately 30 kD (M protein) and 45 kD (nucleocapsid protein, the product of the N gene of APV) were consistently detected by Western blot analysis with sera positive for APV antibodies.

ELISA tests performed on serially diluted field sera from APV-infected flocks produced typical ELISA curves with an endpoint (OD below 0.2) between 1:320 and 1:1280 dilutions (FIG. 1). Since all positive and negative sera gave distinct results at 1:40 dilutions, this dilution was used to analyze serum samples and compare M protein ELISA with ELISA using partially purified APV virions.

A. Specificity of an M Protein ELISA

Sera negative for APV were tested by M protein ELISA. The mean (± SD) absorbance of all negative sera (n = 41) by such an ELISA was 0.088 (± 0.039). Using mean ± 3 standard deviations as the cut-off value (0.2), all the samples were negative using an M protein ELISA and the relative specificity of this ELISA was 100%.

Additionally, M protein ELISA was tested for cross reactivity with other avian pathogenic viruses, and the results are summarized in Table 1. M protein ELISA was positive for 3 different isolates of APV, and negative for several other avian viruses including infectious bursal disease virus, reovirus, avian leukemia virus, avian encephalitis virus, and Newcastle disease virus.
Table 1. Cross-Reactivity of M Protein ELISA with Other Avian Pathogens

<table>
<thead>
<tr>
<th>PATHOGEN</th>
<th>M ELISA OD (1:40)</th>
<th>RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>APV-UK Isolate A</td>
<td>2.167</td>
<td>Positive</td>
</tr>
<tr>
<td>APV-Hungary Isolate B</td>
<td>0.381</td>
<td>Positive</td>
</tr>
<tr>
<td>APV-US Isolate C</td>
<td>1.165</td>
<td>Positive</td>
</tr>
<tr>
<td>Infectious Bursal Disease Virus</td>
<td>0.009</td>
<td>Negative</td>
</tr>
<tr>
<td>Reovirus</td>
<td>-0.044</td>
<td>Negative</td>
</tr>
<tr>
<td>Avian Leukosis Virus</td>
<td>0.13</td>
<td>Negative</td>
</tr>
<tr>
<td>Avian Encephalitis Virus</td>
<td>-0.032</td>
<td>Negative</td>
</tr>
<tr>
<td>Newcastle Disease Virus</td>
<td>0.13</td>
<td>Negative</td>
</tr>
<tr>
<td>Infectious Bronchitis Virus</td>
<td>2.35</td>
<td>Positive</td>
</tr>
</tbody>
</table>

**Example 3—Comparison of M Protein ELISA and Routine APV ELISA**

184 serum samples from turkeys suspected of APV infection were tested using both a routine APV ELISA and an M protein ELISA. A total of 133 serum samples (72.3%) were positive by the M protein ELISA, whereas only 99 (53.8%) were positive by the routine APV ELISA. 12 serum samples were negative by the M protein ELISA but positive by the routine APV ELISA. To determine if these 12 serum samples generated false positive results using the routine APV ELISA, Western blot analysis was performed using either recombinant M protein or partially purified proteins, as described in Example 2. A sample positive by the M protein ELISA was used as a positive control and reacted with the 30 kD M protein in both antigenic preparations. None of the 12 serum samples reacted with either recombinant M protein or APV proteins by immunoblot. In contrast, all samples that were positive by M protein ELISA detected recombinant M protein or APV proteins by Western blot, yielding a sensitivity rating for the routine APV ELISA of only 74% as compared to that of the newly developed M protein ELISA.

The M protein ELISA also gave consistently higher OD readings with convalescent serum samples (n = 34) collected from turkeys experimentally infected with APV 28 days post-infection (FIG. 2; p<0.001, paired t-test). Only 18 samples (54.5%)
were positive using the routine APV ELISA, whereas 33 samples (97.1%) were positive using the M protein ELISA. This indicates that the M protein ELISA is more sensitive than the routine APV ELISA.

Example 4—Cloning and Expression of the APV N Gene

The nucleocapsid (N) gene (nucleotides 14-1198, see GenBank Accession No. AF176590) of the APV/CO isolate was amplified by PCR using primers N1 (5’-ATGTCTCTTCCAGGGATTACAGCTTAG-3’ (SEQ ID NO:4) and N2 (5’-TTACTCATAATCATTCTGGCCTTC-3’ (SEQ ID NO:5)) and the gel-purified PCR product was cloned into a pCR7/NT-TOPO vector (Invitrogen). The pCR7/NT-TOPO vector is designed for the expression of nucleic acids downstream of a T7 promoter and provides for an RGS-6xHis (histidine) tag upstream of the cloning site. A plasmid containing the N1-N2 PCR product cloned in the correct orientation was first passaged through TOP10 F' E. coli cells and then transformed into E. coli BL21(DE3)pLysS cells (Invitrogen). These cells carry the DE3 bacteriophage lambda lysogen having a gene encoding T7 polymerase under control of the lacUV5 promoter and lac repressor. Expression of the N sequences was induced by addition of 0.5 mM IPTG. Treatment with IPTG inactivated the lac repressor and enabled expression of T7 polymerase, which subsequently transcribed the N sequences. The recombinant N polypeptide containing an N-terminal histidine tag was 429 amino acids in length. Recombinant N protein was detected by Western blot using Express-Detector nickel-HRP conjugate (KPL). Maximum expression of recombinant N polypeptide in E. coli was observed 4 h post-induction under native lysis conditions. Recombinant N protein was affinity purified using Ni-NTA agarose (Qiagen Inc.) that binds the histidine affinity tag. To further eliminate contaminating E. coli proteins, recombinant N proteins were resolved on a 10% polyacrylamide gel by discontinuous SDS-PAGE, visualized by Coomassie staining, cut from the gel and homogenized in phosphate buffered saline (pH 7.2).

Example 5—Western Blot Analysis

Recombinant N polypeptide or partially purified APV proteins were resolved in 10% polyacrylamide gel by discontinuous SDS-PAGE and transferred to nitrocellulose
membranes by electroblotting. Membranes were blocked in 5% nonfat dry milk for 1 h at room temperature and incubated for 30 min with a 1:100 dilution of hyperimmune antiserum in blocking solution. After washing 3 times with PBS containing 0.05% Tween 20, membranes were incubated for 30 min with HRP conjugate (KPL). For detection of the histidine tag, membranes (after blocking) were incubated with anti-His HRP conjugate for 15 min. After 3 washings, detection was done by chromogenic 3,3‘,5,5’ tetramethyl benzidine (TMB) membrane peroxidase substrate system (KPL).

**Example 6—Antisera to N Protein**

Rabbit antiserum was produced using gel-purified recombinant N protein. Each rabbit received 3 subcutaneous injections of approximately 100 µg of recombinant protein in Freund’s incomplete adjuvant on days 0, 14 and 28. A final intravenous injection of approximately 100 µg was given on day 35 and rabbits were bled 72 h later. Antiserum was tested by hybridizing to Western blots containing purified APV protein and recombinant N polypeptides as described previously (Gulati et al., *J. Clin. Microbiol.*, 38:4010-14 (2000)). The antiserum specifically detected a 47 kD band from an APV/CO protein preparation or purified recombinant N polypeptide by Western blotting. Hyperimmune serum against the APV/US isolate (APV/MN-1a) was raised in turkeys as previously described (*Current Protocols in Immunology*, Ch. 2 and 8, Coligan et al., eds., John Wiley & Sons (1996)).

**Example 7—N Protein Antibody ELISA**

Alternate rows of ELISA plates (Immulon1B; Dynatech) were coated with 100 µl of rabbit N-specific antiserum or pre-immune rabbit serum diluted 1:1500 in 0.05 M sodium carbonate buffer (pH 9.6) and incubated at 37°C for 2 h. Non-specific binding sites were blocked overnight at 4°C by incubation with 4% fetal horse serum (100 µl) in PBS containing 0.05% Tween-20 (PBS-T). Ni-NTA column-purified N protein (50 µl) appropriately diluted in ELISA dilution/blocking reagent (KPL) was added as antigen and incubated for 1 h at room temperature. The plates were washed 5 times with PBS-T and 50 µl of a 1:40 dilution of turkey sera in ELISA dilution/blocking reagent were added in duplicate wells corresponding to positive and negative rows and incubated for 1 h at room
temperature. After washing 5 times, 50 μl of anti-turkey IgG horseradish peroxidase conjugate (1:1500) diluted in ELISA dilution/blocking reagent was added to each well and incubated for 1 h at room temperature. Following 5 additional washings, 100 μl of the substrate, 0.04% chromogen o-phenylenediamine in citrate phosphate buffer (pH 5.0) containing 0.04% H₂O₂, was added to each well and incubated for 10 min. The reaction was stopped with 25 μl of 2.5 M H₂SO₄. Absorbance values were measured with an ELISA plate reader (Molecular Devices, Sunnyvale, CA). Results were expressed as a difference between the mean absorbance (A₄₉₀/₄₀₅) of each sample in wells coated with N protein-specific serum and wells coated with preimmune serum.

Hyperimmune sera against subgroup A (UK 14/1), subgroup B (Hungarian 657/4) (gifts from Dr. B. Panigrahy, National Veterinary Science Laboratories, Ames, IA) and subgroup C (APV/US/MN-1a, provided by Dr. Shaw, College of Veterinary Medicine, University of Minnesota, St. Paul, MN) also were tested in an N protein antibody ELISA (FIG. 3). N protein antiserum reacted strongly to sera from APV/US isolates using this ELISA when tested at dilutions starting from 1:40 to 1:1280. However, subgroup A and B sera failed to react in this N protein antibody ELISA, indicating that the recombinant N protein recognized by antiserum from infected turkeys is specific to U.S. infections, and hence, U.S. isolates.

N protein antiserum at a dilution of 1:40 was selected for subsequent assays of turkey sera. The cut-off value for a positive test was determined as mean absorbance plus 3 standard deviations for a panel of turkey sera (n=24) negative for APV infection and was calculated to be 0.15. Serum samples from experimental turkeys (n=55) that were free of APV infection and that tested negative for APV antibodies by routine APV ELISA were also negative using the N protein antibody ELISA format, indicating 100% specificity. Serum specimens from experimentally infected turkeys (n=81) at 4 weeks post-infection were positive by routine APV ELISA and also by N protein antibody ELISA format, showing 100% sensitivity relative to routine ELISA. The routine indirect ELISA, employing APV/CO-infected Vero cells as antigen for coating, was performed as previously described (Chiang et al., J. Vet. Diag. Invest., 12:381-4 (2000)).
Example 8—Comparison of N Protein Antibody ELISA and Routine APV ELISA

Routine APV ELISA and the N protein antibody ELISA described in Example 7 were used to test 183 turkey serum samples submitted to the Minnesota Veterinary Diagnostic Laboratory and suspected of APV infection. A total of 85 serum samples were positive by the N protein ELISA and by the routine ELISA. An additional 38 samples were positive by N protein antibody ELISA. Of these 38 samples, 37 were from turkey flocks exhibiting clinical signs of APV disease. Thus, the N protein antibody ELISA was more sensitive than the routine ELISA in detecting antibodies to APV in turkey sera.

Two samples were positive by routine ELISA but were negative by N protein antibody ELISA. The two samples originated from a turkey flock where all the other samples tested (n=8) were negative by routine ELISA. When these two samples were evaluated by Western blot using recombinant N protein or purified APV proteins, neither sample reacted. Thus, N protein antibody ELISA is highly specific for detecting APV antibodies from turkey sera.

Example 9—N Protein Antigen ELISA

Alternate rows of ELISA plates (Immulon1B) were coated with 100 µl of rabbit N-specific antisera and normal rabbit serum diluted 1:100 in 0.05 M carbonate buffer (pH 9.6) by incubating at 37°C for 2 h. Non-specific binding sites were blocked by incubation with 4% fetal horse serum (100 µl) in PBS containing 0.05% Tween-20 overnight at 4°C. Cell lysates of APV-infected Vero cells (50 µl) diluted 10-fold in ELISA dilution/blocking reagent (KPL) were added and incubated for 1 h at room temperature. The APV/CO strain grown in Vero cells was partially purified according to the methods of Goyal et al., 2000, *J. Vet. Diag. Invest.*, 12:166-8. Vero cells carrying APV/CO were titrated by a plaque assay in Vero cells (Goyal et al., 1978, *J. Water Pollut. Contr. Fed.*, 50:2247-56). Plates were washed 5 times with PBS-T. Hyperimmune turkey sera (50 µl) from an MN-1a isolate diluted 1:100 in ELISA dilution/blocking reagent was added in duplicate wells of positive and negative rows and incubated for 1 h at room temperature. After washing 5 times, 50 µl of anti-turkey IgG HRP conjugate (1:1500) diluted in ELISA dilution/blocking reagent was added to each
well and incubated for 1 h at room temperature. Following 5 additional washings, the substrate, 0.04% chromogen o-phenylenediamine in citrate phosphate buffer (pH 5.0) containing 0.04% H₂O₂, was added (100 μl) to each well and incubated for 10 min. Color development was stopped with 25 μl of 2.5 M H₂SO₄. Results were expressed as the difference between the mean absorbance (A₄₉₀/₄₀₅) of each sample in positive N-serum and negative serum coated wells.

The optimum concentration of coating antibody (1:100) and secondary antibody (1:100) was determined by checkerboard titration (Chiang et al., 2000, J. Vet. Diag. Invest., 12:381-4). The corrected ELISA OD value (Absorbance at 490 nm) of each sample was calculated by subtracting the reading of the well coated with normal rabbit sera from the reading of the well coated with N-specific sera. The cut off value for N protein antigen ELISA was determined to be the mean plus 3 standard deviations of turkey sera (n = 24) negative for APV infection and was calculated to be 0.15. The N protein antigen ELISA demonstrated that an APV infection can be detected in infected cells down to 10⁴ pfu/ml.

To detect APV infection in a biological sample, alternate rows of ELISA plates (Immulon1B) are coated with 100 μl of rabbit N-specific antiserum and normal rabbit serum diluted 1:100 in 0.05 M carbonate buffer (pH 9.6) by incubating at 37°C for 2 h. Non-specific binding sites are blocked by incubation with 4% fetal horse serum (100 μl) in PBS containing 0.05% Tween-20 overnight at 4°C. Biological sample preparations are added and incubated for 1 h at room temperature. Plates are washed 5 times with PBS-T. Hyperimmune turkey sera (50 μl) from an MN-1a isolate is diluted 1:100 in ELISA dilution/blocking reagent, added in duplicate wells of positive and negative rows, and is incubated for 1 h at room temperature. After washing 5 times, 50 μl of anti-turkey IgG HRP conjugate (1:1500) diluted in ELISA dilution/blocking reagent is added to each well and incubated for 1 h at room temperature. Following 5 additional washings, the substrate, 0.04% chromogen o-phenylenediamine in citrate phosphate buffer (pH 5.0) containing 0.04% H₂O₂, is added (100 μl) to each well and incubated for 10 min. Color development is stopped with 25 μl of 2.5 M H₂SO₄. Results are expressed as the difference between the mean absorbance (A₄₉₀/₄₀₅) of each sample in positive N-serum and negative serum coated wells.
OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.
WHAT IS Claimed IS:

1. A method for detecting an avian pneumovirus (APV) infection comprising the steps of:
   (i) contacting an isolated APV polypeptide with an avian antibody source under conditions wherein said APV polypeptide binds to an anti-APV polypeptide antibody, if present in said avian antibody source, to form an antibody-polypeptide complex; and
   (ii) detecting the presence or absence of said antibody-polypeptide complex, wherein the presence of said antibody-polypeptide complex correlates with the occurrence of said APV infection.

2. The method of claim 1, wherein said APV infection is from an isolate selected from the group consisting of a European A, a European B, a Colorado, a Minnesota-1a, a Minnesota-1b, and a Minnesota-2a isolate.

3. The method of claim 1, wherein said avian antibody source is isolated from a bird at least 10 days post-infection of said bird with APV.

4. The method of claim 1, wherein said isolated polypeptide is encoded by an APV gene selected from the group consisting of an N, P, M, F, M2, L, SH and G gene.

5. The method of claim 1, wherein said isolated polypeptide is encoded by an APV M gene.

6. The method of claim 5, wherein said M gene is derived from an APV isolate selected from the group consisting of a European A, a European B, a Colorado, a Minnesota-1a, a Minnesota-1b, and a Minnesota-2a isolate.
7. The method of claim 1, wherein said isolated polypeptide is a fragment of a full-length M polypeptide.

8. The method of claim 7, wherein said fragment comprises the amino acid sequence of SEQ ID NO:1.

9. The method of claim 1, wherein said isolated polypeptide is encoded by an APV N gene.

10. The method of claim 9, wherein said N gene is derived from an APV isolate selected from the group consisting of a European A, a European B, a Colorado, a Minnesota-1a, a Minnesota-1b, and a Minnesota-2a isolate.

11. The method of claim 9, wherein said N gene is derived from an APV isolate selected from the group consisting of a Colorado, a Minnesota-1a, a Minnesota-1b, and a Minnesota-2a isolate.

12. The method of claim 1, wherein said isolated polypeptide is a fragment of a full-length N polypeptide.

13. The method of claim 12, wherein said fragment comprises the amino acid sequence of SEQ ID NO:2.

14. The method of claim 1, wherein said avian antibody source is obtained from a wild bird.

15. The method of claim 1, wherein said avian antibody source is obtained from a domesticated bird.

16. The method of claim 15, wherein said domesticated bird is selected from the group consisting of chickens, turkeys, geese, and ducks.
17. The method of claim 16, wherein said domesticated bird is a turkey.

18. The method of claim 1, wherein said isolated APV polypeptide adheres to a second anti-APV polypeptide antibody.

19. The method of claim 18, wherein said second anti-APV polypeptide antibody is derived from an animal species other than the animal species from which said avian antibody source originates.

20. The method of claim 19, wherein said second anti-APV polypeptide antibody is derived from a non-avian animal species.

21. The method of claim 1, wherein said isolated APV polypeptide is immobilized on a solid substrate.

22. The method of claim 21, wherein said solid substrate is selected from the group consisting of a dipstick, a microtiter plate, a bead, an affinity column, and an immunoblot membrane.

23. The method of claim 1, wherein immunoglobulins present in said avian antibody source are immobilized on a solid substrate.

24. The method of claim 1, wherein said detecting step comprises performing an enzyme-linked immunoassay, a radioimmunoassay, an immunoprecipitation, a fluorescence assay, a chemiluminescent assay, an immunoblot assay, or a particulate based assay.

25. The method of claim 1, wherein said detecting step comprises:

(i) contacting said antibody-polypeptide complex with an indicator molecule that selectively binds to said anti-APV polypeptide antibody; and
(ii) detecting the presence or absence of said indicator molecule.

26. An article of manufacture for detecting anti-APV polypeptide antibodies in an avian antibody source, said article of manufacture comprising an isolated APV polypeptide.

27. The article of manufacture of claim 26, said article of manufacture comprising two or more isolated APV polypeptides, wherein said isolated polypeptides are encoded by different APV genes.

28. The article of manufacture of claim 26, said article of manufacture further comprising an indicator molecule.

29. The article of manufacture of claim 28 wherein said indicator molecule selectively binds to anti-APV polypeptide antibodies produced by the bird species from which said avian antibody source is obtained.

30. The article of manufacture of claim 26, said article of manufacture further comprising reagents for detecting a plurality of avian viral infections.

31. The article of manufacture of claim 30, wherein said plurality of avian viral infections is caused by APV and at least one virus selected from the group consisting of Avian Encephalitis virus, Avian Influenza, Avian Leukosis, Fowl Pox, Infectious Bronchitis Virus, Infectious Bursal Disease Virus, Newcastle Disease virus, and Reovirus.

32. The article of manufacture of claim 26, wherein said isolated APV polypeptide is encoded by a gene from an APV isolate selected from the group consisting of a European A, a European B, a Colorado, a Minnesota-1a, a Minnesota-1b, and a Minnesota-2a isolate.
33. The article of manufacture of claim 26, wherein said isolated APV polypeptide is encoded by an APV gene selected from the group consisting of an N, P, M, F, M2, L, SH and G gene.

34. The article of manufacture of claim 26, wherein said isolated APV polypeptide is encoded by an APV M gene.

35. The article of manufacture of claim 26, wherein said isolated APV polypeptide is a fragment of a full-length M polypeptide.

36. The article of manufacture of claim 35 wherein said fragment comprises the amino acid sequence of SEQ ID NO:1.

37. The article of manufacture of claim 26, wherein said isolated APV polypeptide is encoded by an APV N gene.

38. The article of manufacture of claim 26, wherein said isolated APV polypeptide is a fragment of a full-length N polypeptide.

39. The article of manufacture of claim 38, wherein said fragment comprises the amino acid sequence of SEQ ID NO:2.

40. The article of manufacture of claim 26, wherein said APV polypeptide is immobilized on a solid substrate.

41. The article of manufacture of claim 40, wherein said solid substrate is selected from the group consisting of a dipstick, a microtiter plate, a bead, an affinity column, an immunoblot membrane, and an immunoblot paper.

42. The article of manufacture of claim 26, wherein said isolated APV polypeptide binds to an anti-APV polypeptide antibody from a wild bird.
43. The article of manufacture of claim 26, wherein said isolated APV polypeptide binds to an anti-APV polypeptide antibody from a domesticated bird.

44. The article of manufacture of claim 43, wherein said domesticated bird is selected from the group consisting of chickens, turkeys, geese and ducks.

45. The article of manufacture of claim 44, wherein said domesticated bird is a turkey.

46. The article of manufacture of claim 26, further comprising a presenting molecule.

47. The article of manufacture of claim 46, wherein said presenting molecule and said APV polypeptide are provided as a mixture.

48. A purified antibody having specific binding affinity for an M polypeptide.

49. A purified antibody having specific binding affinity for an N polypeptide.

50. A method for detecting an APV infection comprising the steps of:
   (i) providing a mixture comprising a presenting molecule and an APV polypeptide,
   (ii) contacting said mixture with an avian antibody source under conditions wherein said mixture binds to an anti-APV polypeptide antibody, if said anti-APV polypeptide antibody is present in said avian antibody source, to form an antibody-mixture complex; and
   (iii) detecting the presence or absence of said antibody-mixture complex, wherein the presence of said antibody-mixture complex correlates with the occurrence of an APV infection.
51. The method of claim 50, wherein said presenting molecule is a second anti-APV polypeptide antibody.

52. The method of claim 51, wherein said second anti-APV polypeptide antibody is derived from an animal species other than the animal species from which said avian antibody source originates.

53. The method of claim 52, wherein said second anti-APV polypeptide antibody is derived from a non-avian animal species.

54. The method of claim 50, wherein said APV polypeptide is an isolated APV polypeptide.

55. The method of claim 54, wherein said APV polypeptide is a recombinant APV polypeptide.

56. The method of claim 54, wherein said APV polypeptide is an isolated APV polypeptide from Vero cells.

57. The method of claim 50, wherein said APV polypeptide is encoded by an APV gene selected from the group consisting of an N, P, M, F, M2, L, SH and G gene.

58. The method of claim 50, wherein said APV polypeptide is encoded by an APV M gene.

59. The method of claim 50, wherein said APV polypeptide is encoded by an APV N gene.

60. The method of claim 50, wherein said APV infection is by an APV U.S. isolate.
61. The method of claim 60, wherein said APV U.S. isolate is selected from
the group consisting of a Colorado, a Minnesota-1a, a Minnesota-1b, and a Minnesota-2a
isolate.

62. The method of claim 50, wherein said avian antibody source is isolated
from a bird at least 10 days post-infection of said bird with APV.

63. The method of claim 50, wherein said avian antibody source is obtained
from a domesticated bird.

64. The method of claim 63, wherein said domesticated bird is selected from
the group consisting of chickens, turkeys, geese, and ducks.

65. The method of claim 62, wherein said domesticated bird is a turkey.

66. The method of claim 50, wherein said presenting molecule is immobilized
on a solid substrate.

67. The method of claim 66, wherein said solid substrate is selected from the
group consisting of a dipstick, a microtiter plate, a bead, an affinity column, and an
immunoblot membrane.

68. The method of claim 50, wherein said mixture is immobilized on a solid
substrate.

69. The method of claim 50, wherein immunoglobulins present in said avian
antibody source are immobilized on a solid substrate.

70. The method of claim 50, wherein said detecting step comprises performing
an enzyme-linked immunosassay, a radioimmunosassay, an immunoprecipitation, a
fluorescence assay, a chemiluminescent assay, an immunoblot assay, or a particulate
based assay.

71. The method of claim 50, wherein said detecting step comprises:
(i) contacting said mixture-antibody complex with an indicator
molecule that selectively binds to said mixture-antibody complex; and
(ii) detecting the presence or absence of said indicator molecule.

72. A method for detecting an APV infection comprising the steps of:
(i) contacting a presenting molecule with a biological sample under
conditions wherein said presenting molecule binds to an APV polypeptide, if said APV
polypeptide is present in said biological sample, to form a presenting molecule-APV
polypeptide mixture,
(ii) contacting said mixture with a mixture-recognizing molecule to
form a complex; and
(iii) detecting the presence or absence of said complex, wherein the
presence of said complex correlates with the occurrence of an APV infection.

73. The method of claim 72, wherein said biological sample is isolated from a
bird at least 4 days post-infection of said bird with APV.

74. The method of claim 72, wherein said presenting molecule is immobilized
on a solid substrate.

75. The method of claim 72, wherein said mixture-recognizing molecule is
immobilized on a solid substrate.

76. The method of claim 72, wherein said detecting step comprises:
(i) contacting said complex with an indicator molecule that selectively
binds to said mixture-recognizing molecule; and
(ii) detecting the presence or absence of said indicator molecule.
77. An isolated APV polypeptide encoded by an APV N gene.

78. An isolated APV polypeptide encoded by an APV M gene.
Figure 1

The graph illustrates the results of Routine APV-ELISA and M protein ELISA for 34 samples. The x-axis represents the absorbance at 450 nm, ranging from 0 to 1.2. The y-axis lists the samples (n=34). The bars indicate the absorbance values for each sample, with different symbols representing the two ELISA methods.
Figure 2

[Graph showing data for positive and negative sera (pooled)].
Figure 3