Journal of Veterinary Diagnostic Investigation

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What is This?

Simultaneous detection of five major serotypes of *Avian coronavirus* by a multiplex microsphere-based assay

Ha-Jung Roh, Deborah A. Hilt, Mark W. Jackwood[1]

Abstract. *Avian coronavirus* (commonly known as Infectious bronchitis virus [IBV]) is of major economic importance to commercial chicken producers worldwide. Due to the existence of multiple serotypes and variants of the virus that do not cross-protect, it is important to diagnose circulating serotypes and choose the right vaccine type for successful protection. In an effort to improve conventional diagnostic tests, a microsphere-based assay was developed and evaluated for simultaneous detection of the most common IBV vaccine serotypes in the United States: Arkansas (Ark), Connecticut (Conn), Massachusetts (Mass), Delaware (DE072), and Georgia 98 (GA98). The analytical specificity and sensitivity, and diagnostic specificity and sensitivity, were evaluated. The microsphere-based assay was highly specific to designated serotypes and generated reproducible data. Comparing the microsphere-based assay to nucleotide sequencing, the 2 methods agreed more than 93% (kappa value > .77). In addition, the microsphere-based assay could detect coinfections in clinical samples. The results demonstrate the utility of the microsphere-based assay as a rapid and accurate diagnostic tool with the potential for high throughput diagnosis.

Key words: Arkansas (Ark); *Avian coronavirus*; Connecticut (Conn); Delaware 072 (DE072); diagnosis; Georgia 98 (GA98); Infectious bronchitis virus; Massachusetts (Mass); microsphere-based assay; multiplex; serotyping.

Introduction

Avian coronavirus (commonly known as Infectious bronchitis virus [IBV]; order *Nidovirales*, family *Coronaviridae*, subfamily *Coronavirinae*, genus *Gammacoronavirus*) is a positive-sense single-stranded RNA virus with a genome ranging from 27 to 28 kb. The virus is highly contagious and primarily infects epithelial cells in the upper respiratory tract of chickens, but can also infect epithelial cells in the kidney, oviduct, testes, and alimentary tract.[2] Infectious bronchitis is an economically important disease that affects chickens, which are the primary host, with infections also reported in ring-necked pheasants (*Phasianus colchicus*)[11] and peafowl (order *Galliformes*).[26] The virus causes an upper respiratory disease in young chickens and can cause decreased egg production and egg quality in hens.[6,19] Some strains of IBV target the kidneys causing an interstitial nephritis.[29,33] Since the first identification of IBV in the 1930s in the United States,[10] many serotypes and variants have been identified worldwide. Differing serotypes do not confer cross-protection,[7] which makes it extremely difficult to control the disease.[3] Antigenic variation of IBV is largely due to mutations and recombination that affect the spike protein. Spike proteins are made up of 2 subunits (S1 and S2) and form club-shaped projections that extend from the surface of the virus particle. The S1 subunit, which makes up the terminus of the spike, contains epitopes that induce neutralizing antibodies. Mutations in the S1 gene that change the epitopes can result in virus particles escaping the immune response.[3] Currently, live attenuated vaccines are in use for control of IBV in broilers.[2] Because of the poor cross-protection between heterologous serotypes, diagnosing circulating viruses in the field and choosing antigenically homologous vaccine strains are critical steps for the successful control of IBV.

Conventional diagnostic methods to differentiate IBV serotypes include virus isolation in specific pathogen–free (SPF) embryonated eggs followed by virus neutralization (VN) tests, hemagglutination inhibition (HI) tests, or antigen-capture enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies.[1,12,32] However, genetic-based tests to identify IBV types have become the test of choice since the discovery that sequences in the S1 gene are correlated with different serotypes of IBV.[15,18,28] Reverse transcription polymerase chain reaction (RT-PCR), targeting the S1 portion of the spike protein, followed by sequencing of the RT-PCR product,[25] restriction enzyme fragment length polymorphism (RFLP),[23,24] or hybridization with IBV-specific probes[14,17,22]

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have been developed for differentiating serotypes and variants of the virus.

Microsphere-based suspension array[a] is a relatively new diagnostic platform that enables high throughput detection of nucleic acids as well as other analytes. Microspheres contain 2 internal fluorochromes with different intensities, giving each microsphere a unique spectral character. This unique spectral character theoretically allows up to 500 different microspheres to be combined and used in the microsphere-based assay.[8] The test involves direct hybridization between PCR-amplified DNA products from clinical samples and targets specific oligonucleotide probes coupled to the microspheres. The amplified products are conjugated with reporter dyes at the 5'- or 3'-ends. A microsphere analyzer uses lasers to excite the internal dyes of the microsphere and the reporter dye conjugated to PCR products, and reads the fluorescent levels. The result is reported as median fluorescence intensity (MFI) identifying the microsphere spectral address and the presence of the PCR-amplified product.[8] Microsphere-based assays have been used for the diagnosis of many infectious pathogens including, but not limited to *Salmonella*,[9] avian influenza,[20] and human respiratory disease–associated viruses.[27] The majority of IBVs isolated from commercial chickens are vaccine-type viruses and rapidly distinguishing them from each other and from variant viruses is critical for control of infectious bronchitis. In the current study, a multiplex microsphere-based assay for typing the 5 major IBV vaccine viruses used in the United States has been developed and validated. The analytical sensitivity and specificity of the multiplex microsphere-based assay was analyzed, and evaluation of the assay as a potential diagnostic tool for IBV was performed using previously identified clinical samples. The results were compared to current tests utilizing RT-PCR amplification and nucleotide sequencing.

Materials and methods

Virus samples

For initial assay development and optimization, previously identified virus stocks were used. Arkansas Ark/ArkDPI/81 (Ark-DPI), Massachusetts Mass/Mass41/41 (Mass41), Connecticut Conn/Conn46/51 (Conn), Delaware DE/DE072/92 (DE 072), and Georgia 98 GA/GA98/0470/98 (GA98) were propagated in 9- to 10-day-old SPF embryonated chicken eggs, and the 50% embryo infectious dose (EID_{50}) titer was calculated by the Reed and Muench method.[30] Other chicken respiratory pathogens, which include *Newcastle disease virus* (NDV), *Gallid herpesvirus 1* (commonly known as Infectious laryngotracheitis virus [ILTV]), and avian *Influenza A virus* (AIV), as well as *Mycoplasma gallisepticum* (MG) used in the study were obtained from the Poultry Diagnostic and Research Center, Athens Georgia.

Design of primers and serotype-specific probes

Primers and probes were designed against a hypervariable region in the IBV S1 gene for the Ark-DPI, Mass41, and Conn viruses. Because DE072 and GA98 are antigenically related and show high sequence similarity in the S1 gene, a single probe was designed to detect both serotypes (Table 1). Currently available sequences of IBVs in GenBank were used and aligned using commercial software,[b] and regions specific to each serotype were identified and used to develop serotype-specific probes. Forward and reverse primers were designed in conserved areas flanking the probes. The amplified product is approximately 537 bp in length. Basic Local Alignment Search Tool (BLAST) analysis was performed (blast.ncbi.nlm.nih.gov) to verify the specificity of probes and primers. The primers and probes were synthesized by a commercial company.[c] The serotype-specific probes contain an amino-modified 6-carbon spacer at the 5'-end for binding to the beads. The forward RT-PCR primer included a 5'-biotinylation modification for binding to the reporter dye. In addition, biotinylated oligonucleotides (antiprobes) complementary to the probes bound to the microspheres were obtained to evaluate binding of the probes to the microspheres.

RNA extraction and RT-PCR amplification

Viral RNA was extracted from 200 μl of allantoic fluid or clinical samples according to the manufacturer's instructions[d] and eluted into 50 μl of buffer. Extracted RNA was stored at –80°C. The RT-PCR reaction was performed using a commercially available RT-PCR mix following the manufacturer's instructions.[e] The RT-PCR reaction mixture included 10 μl of 5× RT-PCR reaction buffer, 10 mM of each deoxyribonucleotide triphosphate, 12.5 μM of each primer, 40 U of RNase inhibitor,[f] 2 μl of dithiothreitol, 2 μl of $MgCl_2$, 1 μl of enzyme mix, and 5 μl of extracted RNA. The RT-PCR reaction was performed on a thermocycler[g] using the following conditions: 1 cycle of 42°C for 60 min and 95°C for 5 min; 10 cycles of 94°C for 30 sec, 53°C for 30 sec, and 68°C for 90 sec; 25 cycles of 94°C for 30 sec, 53°C for 30 sec, and 68°C for 90 sec adding 5 sec per cycle; and final extension at 68°C for 7 min. For the limit of detection test, RT-PCR products were agarose gel purified using a commercially available kit according to the manufacturer's instructions.[h] For other tests including specificity and clinical sample evaluation, RT-PCR products were used directly without agarose gel purification.

Microsphere-based assay

Coupling of serotype-specific probes and polystyrene microspheres was performed according to the manufacturer's bead coupling protocol.[i] A 10-μl aliquot of biotinylated RT-PCR

product was added directly to 33 μl of working microsphere mixture (single or multiple microspheres) in 7 μl of Tris–ethylenediamine tetra-acetic acid buffer (pH 8.0) in a single tube. Hybridization was performed at 95°C for 5 min followed by 55°C for 15 min. After hybridization, the mixtures were centrifuged at 2,250 × g for 3 min, and the pellet was resuspended in 1× tetramethylammonium chloride containing 3 μl/ml of streptavidin-R-phycoerythrin[j] followed by a 5-min dark incubation. The beads hybridized with amplified RT-PCR product were analyzed using a microsphere reader,[k] and the signal was expressed as MFI. A MFI signal greater than triple the highest background MFI for a given microsphere set was considered positive. Background fluorescence was determined using negative controls, which were included in all assays. Multiple positive (Ark-DPI, Mass41, Conn, DE072, and GA98 positive controls) and negative controls (bead control, RT-PCR control) were included in each run.

Specificity and limit of detection of the microsphere-based assay

To determine specificity of the microsphere-based assay, single-plex and multiplex assays were performed in triplicate with RNA extracted from known positive allantoic fluid samples of different IBV serotypes as well as with nucleic acid extracted from samples containing other avian respiratory pathogens, including AIV, NDV, ILTV, and MG. Extracted RNA from samples was processed as described above.

The limit of detection for each given microsphere set was determined using 2-fold serial dilutions of gel-purified biotinylated RT-PCR products. Purified DNA concentrations were determined using a spectrophotometer.[l] Single-plex and multiplex microsphere-based assays were performed in triplicate, and the limit of detection was determined by the lowest dilution giving positive MFI. Corresponding DNA amplicon copy numbers were calculated based on the assumption of the average weight of a base pair (bp) is 650 Daltons using the following equation: [no. of copies = (DNA amount (ng) × 6.022×10^{23})/(length of template (bp) × (1×10^9) × 650)].

Evaluation of the microsphere assay

For evaluation of the assay, 59 clinical samples were collected from chickens.[m] Clinical samples were previously identified by RT-PCR followed by nucleotide sequencing. The multiplex microsphere-based assay was performed using RNA extracted from clinical samples, and test results were compared to the sequencing results.

RFLP analysis

RNA extracted from clinical samples was used to amplify the entire S1 gene region by RT-PCR using previously published primers[15,24] (NEWS1OLIGO5': 5'-TGAAAACTGAAC AAAAGAC-3', Degenerate3': 5'-CCATAAGTAACATA AGGRCRA-3'). The RT-PCR conditions were previously described.[23] The S1 gene RT-PCR products were gel purified using a commercially available kit[h] and digested using restriction endonucleases (*Bst*YI, *Hae*III, and *Xcm*I) according to the manufacturer's recommendation.[n] Digested samples were electrophoresed on 2% agarose gels, and the restriction fragment length patterns of the samples were analyzed and compared to the reference viruses.

Results

Confirmation of bead coupling and selection of serotype-specific probes

The coupling of probes to each set of microspheres was evaluated using biotinylated oligonucleotides (antiprobes) complementary to the probes. The antiprobe was serially diluted (in the range of 5–200 fmol), and the MFI of each reaction was compared. The MFI of the antiprobe increased linearly with the quantity of antiprobe in the sample (data not shown). To verify the specificity of the probes for the designated IBV serotypes, single-plex assays were performed, in triplicate, using the amplicons generated from each reference virus. Data for the probes that only detected the targeted IBV serotypes with no cross-reactivity are shown in Figure 1A. The probes were designated as Ark-P, Mass-P, Conn-P, and Del/GA98-P, with the Del/GA98-P designed to detect both DE072 and GA98 serotypes. Background fluorescence was determined using negative controls, which were included in all assays.

Specificity of the multiplex microsphere-based assay

The specificity of the multiplex microsphere-based assay was examined by testing 17 different pathogens, including different serotypes of IBV, and the results are shown in Table 2. As shown in Figure 1 and Table 2, the assay was able to detect the target IBV serotypes, whereas nonspecific binding to other IBV serotypes and pathogens was not detected.

Limit of detection of the microsphere-based assay

The limit of detection of the single-plex and the multiplex assay for biotinylated amplicons generated using RT-PCR was estimated by analyzing 2-fold dilutions of amplified S1 gene products from Ark-DPI, Mass41, Conn, DE072, and GA98. In addition, corresponding DNA amplicon copy numbers were calculated, and the data are presented in Table 3.
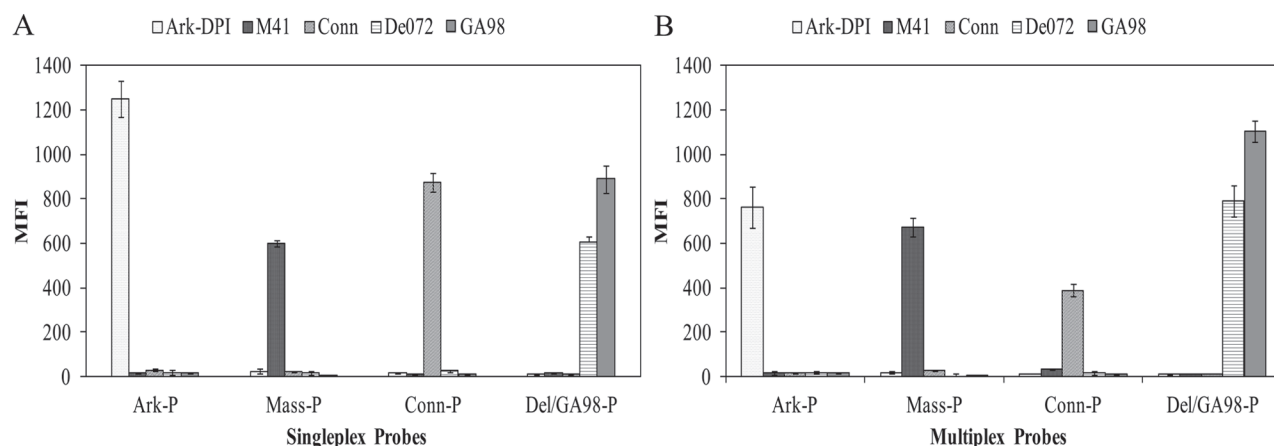
Figure 1. Target serotype specificity of the probes used in the current study in a single-plex (**A**) and a multiplex (**B**) microsphere-based assay. The median fluorescence intensity (MFI) was measured in triplicate, and the error bars indicate means ± standard deviations.

Table 1. Primers and probes used in the current study.

Primer/probe	Target	Nucleotide sequence (5'→3')	5' modification
IBV-F'-Bio	Universal	5'-TAGTCACYMTTTTGTKTGCACTA*-3'	Biotin
IBV-R'-537		5'-TTAGANGTRWAAASRAGRTYRCCATTTAA-3'	
Ark-P	Arkansas	5'-CACAAAAGATTCGTTGTCATATAAATT-3'	NH_2-C$_6$'
Mass-P	Massachusetts	5'-AGGTGAAGAGCCTGCATTATTAGATTC-3'	NH_2-C$_6$'
Conn-P	Connecticut	5'-ACCAATAATACCAACAATACACTCTCTTAA-3'	NH_2-C$_6$'
Del/GA98-P	Delaware 072 and GA98	5'- ACTATGCAAYTATGACCRGTTCCACCAC-3'	NH_2-C$_6$'

* Degenerate primer abbreviations are as follows: Y, C, or T; M, C, or A; K, T, or G; N, all; R, A, or G; W, A, or T; S, G, or C.

Reproducibility of the multiplex microsphere-based assay

To confirm the reproducibility of the multiplex microsphere-based assay, intra-assay (the individual test results within a single run) and interassay (the individual test results from one run to another) variability was evaluated. For each probe with each targeted reference virus, the coefficient of variation of MFI values within a single run (intra-assay) ranged from 0.03 to 0.05, and that of interassay ranged from 0.01 to 0.08 (data not shown).

Evaluation of clinical samples

To evaluate the performance of the assay as a diagnostic tool, 59 clinical samples were tested without prior knowledge of their type using the multiplex microsphere-based assay. Clinical samples consisting of tracheal swabs were collected from chickens, and nucleotide sequencing was used to identify the IBV type in each sample. Data from the microsphere-based assay and nucleotide sequencing are compared in Table 4. Based on the 59 clinical samples tested, the specificities of detection for the different serotypes of IBV were 88.8% (Ark-P), 93.2% (Mass-P), 100% (Conn-P), and 96.6% (Del/GA98-P), and the sensitivities were 100% for all targeted serotypes. In addition, 7 samples were identified as coinfections of 2 different serotypes of IBV by the multiplex microsphere-based assay (Table 5). To verify that the samples indeed contained 2 different IBV types, RFLP analysis was conducted on the amplified S1 gene and both Ark-DPI and Mass41 type viruses were found to be present in clinical sample 82323, which was only identified as Ark serotype–positive by nucleotide sequencing. In addition, sample 82427 was also determined to be a coinfection of Mass41 and DE072/GA98 type viruses by RFLP analysis (data not shown). The other samples where a weak MFI signal was observed could not be confirmed as containing 2 IBV types by RFLP analysis. Agreement between the multiplex microsphere-based assay and the nucleotide sequencing results for all tested viruses was >93% (kappa correlation > .77; Table 4).

Table 2. Analytic specificity of multiplex microsphere-based assay against pathogens used in the current study.*

Virus/bacteria	Subtype/serotype/species	Ark-P	Mass-P	Conn-P	Del/GA98-P
		Multiplex probe specificity			
IBV	Ark-DPI	+	−	−	−
	Mass41	−	+	−	−
	Conn	−	−	+	−
	DE072	−	−	−	+
	GA98	−	−	−	+
	GA08	−	−	−	−
	Iowa	−	−	−	−
	Florida	−	−	−	−
	JMK	−	−	−	−
	Gray	−	−	−	−
	Holte	−	−	−	−
	Ca99	−	−	−	−
NDV	Lasota type	−	−	−	−
ILTV	USDA†	−	−	−	−
AIV	H5N2	−	−	−	−
	H7N3	−	−	−	−
Mycoplasma	*Mycoplasma gallisepticum*	−	−	−	−

* IBV = Avian coronavirus (commonly known as Infectious bronchitis virus); NDV = *Newcastle disease virus*; ILTV = *Gallid herpesvirus 1* (commonly known as Infectious laryngotracheitis virus); AIV = avian *Influenza A virus*.
† AviServe ILT strain.

Table 3. Limit of detection of the single-plex and multiplex microsphere-based assay.*

Probe	Target	DNA amount (ng)	Corresponding copy number †
Ark-P			
Single-plex	Ark-DPI	6.4	1.10×10^{10}
Multiplex	Ark-DPI	9.05	1.56×10^{10}
Mass-P			
Single-plex	Mass41	3.75	6.46×10^{9}
Multiplex	Mass41	4.2	7.24×10^{9}
Conn-P			
Single-plex	Conn	7.5	1.29×10^{10}
Multiplex	Conn	10.5	1.81×10^{10}
Del/GA98-P			
Single-plex	DE072	15.9	2.74×10^{10}
	GA98	7.2	1.24×10^{10}
Multiplex	DE072	15.75	2.71×10^{10}
	GA98	7.2	1.24×10^{10}

* Limit of detection was estimated using reverse transcription polymerase chain reaction and was estimated by analyzing 2-fold dilutions of amplified S1 gene products from Ark-DPI, Mass41, Conn, DE072, and GA98.
† Corresponding copy number was calculated using the following equation: (1 bp = 650 Dalton)
No. of copies = [DNA amount (ng) × (6.022×10^{23})]/[(length of template (bp)) × (1×10^{9}) × 650].

Conventional versus microsphere-based assays

In Figure 2, the procedures and timelines for conventional IBV serotype identification assays and for the microsphere-based assay are compared. The VN test, a traditional method for serotyping IBV,[19] takes an average of 7 days from incubation to data analysis. Extraction of RNA and RT-PCR steps are common to nucleotide sequencing and microsphere-based assays. These common steps aside, nucleotide sequenc- ing requires 1–2 additional days to acquire sequencing data, whereas the microsphere-based assay takes less than 5 hr including RNA extraction and RT-PCR.

Discussion

Infectious bronchitis virus is one of the most important patho- gens in chickens causing a significant negative economic

Table 4. Comparison of the multiplex microsphere-based assay with nucleotide sequencing analysis of clinical samples.*

| Type-specific probe	Microsphere-based assay results	Sequencing results		Sensitivity (%)	Specificity (%)	Agreement (%)	Kappa value
		Positive	Negative				
Ark-P	+	23	4†	100	89.1	93.2	.86
	−	0	32				
Mass-P	+	9	4†	100	92	93.2	.77
	−	0	46				
Conn-P	+	5	0	100	100	100	1.00
	−	0	54				
Del/GA98-P	+	10	2	100	95.9	96.6	.88
	−	0	47				

* Clinical samples were previously confirmed by nucleotide sequencing.
† Samples with double positive signals from the microsphere-based assay including GA08 were considered.

Table 5. Clinical samples with double signals of microsphere-based assay.

Sample ID	Sequencing results	Microsphere-based assay results				RFLP results
		Ark-P	Mass-P	Conn-P	Del/GA98-P	
67297	GA08	Weak positive*	Weak positive	Negative	Negative	GA08
67634	GA08	Weak positive	Weak positive	Negative	Negative	GA08
82323	Ark	Positive	Positive	Negative	Negative	Ark-DPI and Mass41
82427	Mass	Negative	Positive	Negative	Positive	Mass41 and DE072/GA98
83147	Ark	Positive	Negative	Negative	Weak positive	Ark-DPI
83188	GA08	Weak positive	Weak positive	Negative	Negative	GA08
87828	Conn	Weak positive	Negative	Positive	Negative	Conn

* Positive median fluorescence intensity value was considered as weak positive when it was less than 3 folds of cutoff value. RFLP = restriction enzyme fragment length polymorphism.
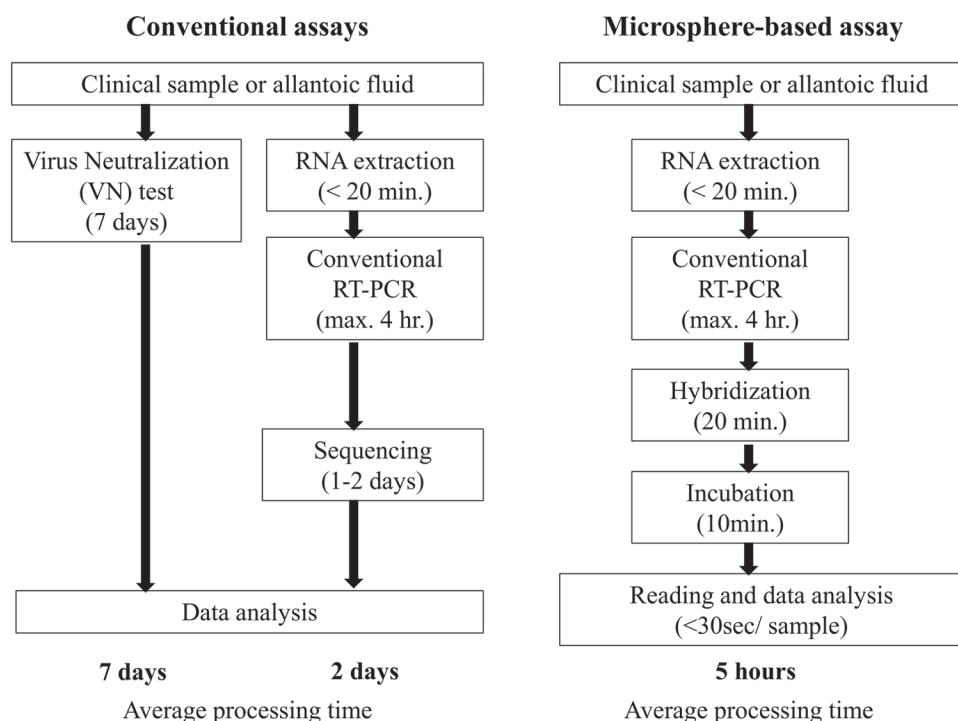
Figure 2. Comparison of the procedures and timelines of conventional assays and the microsphere-based assay for Infectious bronchitis virus serotype identification.

impact on the poultry industry worldwide. Infection with IBV can be moderated by vaccination, but due to its numerous serotypes and variants that do not cross-protect, constant surveillance of circulating viruses are needed so that the appropriate vaccine can be selected. The VN test is the definitive serotype identification test but an increasing number of IBV variants make it almost impossible to conduct the VN test for all possible serotypes.[7] The alternatives to traditional serotyping tests are molecular methods that identify virus genotype such as RFLP analysis and nucleotide sequencing of the RT-PCR–amplified S1 gene.

In the current study, a multiplex microsphere-based assay to identify 4 major IBV serotypes commonly used as vaccines was developed. Distinctive serotypes of IBV are associated with differences in the sequence of the S1 glycoprotein, and unique hypervariable regions have been previously identified[4,21] and correlated with different types of the virus.[5,18,31] The microsphere assay described in the current study also targets the S1 hypervariable region. Universal primers were designed based on conserved sequences, and serotype-specific probes were designed to anneal to the hypervariable regions allowing the identification of 4 different genetic types (Ark-DPI, Mass41, Conn, and DE072/GA98). The DE072 and GA98 virus types share high antigenic similarity provide significant cross-protection[13] and have limited variability in the S1 hypervariable region. The DE072 and GA98 strains were both included in evaluation of the assay because both strains are used as vaccines in the field, and it is important to document that both would be detected by the assay.

The IBV type–specific probes were tested against reference viruses to ensure no cross reactivity occurred with nontargeted IBV serotypes as well as other selected avian upper respiratory tract pathogens. Different serotypes of IBV are due to antigenic variation in the spike protein, which is largely due to mutations and recombination.[16] Because this assay did not detect nontargeted IBV serotypes (IBV types with genetically different spikes) it is likely that genetic mutations and/or recombination affecting spike and resulting in a different serotype would yield the expected negative result.

The analytical sensitivity of the probes for the reference viruses in single-plex and multiplex assays ranged from 3.75 to 15.9 ng, corresponding to 6.46×10^9 to 2.74×10^{10} genome copy numbers. Under multiplex conditions, the sensitivity of the probe was slightly lower.

A total of 59 clinical samples was examined, and no statistically significant differences between the microsphere-based assay and nucleotide sequencing (>93.2% agreement for all of the probes) were found. The sensitivity of each probe against its target IBV type was 100%, and the specificity was >89.1%. The kappa correlation values between the 2 methods were higher than .77, which indicates the microsphere-based assay is as specific as the nucleotide sequencing method.

One unexpected finding from the analysis of clinical samples was that 2 different IBV types were identified by the microsphere assay in samples previously identified by sequencing as having only 1 IBV type. Typically when more than 1 IBV type is present in a sample, the sequencing data are not readable. However, nucleotide sequencing detected Ark-DPI for clinical sample 82323 and Mass41 for clinical sample 82427, whereas the microsphere-based assay detected Ark-DPI and Mass41 for clinical sample 82323 and Mass41 and DE072 for clinical sample 82427. RT-PCR amplification of the S1 gene was used, followed by RFLP analysis to confirm these results. Typically, multivalent IBV vaccines are given to commercial poultry to provide a broad immune response. Thus, the presence of more than 1 IBV type in a clinical sample is not uncommon. Detecting multiple IBV types in a single sample is a distinct advantage of the microsphere-based assay over nucleotide sequencing.

Three of the clinical samples (67297, 67634, and 83188) previously identified as the GA08 IBV type by nucleotide sequencing were expected to be negative in the microsphere-based assay, but instead all 3 were weakly positive for Ark-DPI and Mass41. When confirmation of the presence of Ark-DPI and Mass41 in those samples using RFLP was attempted, only the GA08 type of IBV was detected. Tests with the Ark-P and Mass-P probes showed that they did not cross-react with the GA08 virus (Table 2) so it is possible that a low level of Ark-DPI and Mass41 could indeed be present but undetectable in those samples by sequencing or RFLP analysis. It is interesting to note that those clinical samples were obtained from chickens previously given live attenuated Ark-DPI and Mass41 vaccines. However, it is also possible that the weak-positive signals obtained with the microsphere-based assay, could be false-positive results.

The current study demonstrated that the microsphere-base assay including RNA extraction and RT-PCR steps requires less than 5 hr from start to finish, providing same-day results. In addition, procedures such as RNA extraction and washing between hybridization and incubation steps can easily be automated to further reduce handling time and manipulation errors. In addition to automation, adaptability to high throughput format as well as increased flexibility by introducing a 96-well plate format and more probes is possible. This is all in addition to the most significant advantage of the test, which is multiplexing to identify more than 1 IBV type in a single sample.

In conclusion, a multiplex microsphere-base assay targeting the hypervariable S1 gene region to identify commonly used IBV vaccine serotypes was developed. The assay is comparatively rapid, is specific, and correlates well with conventional identification methods with the advantage that it can detect more than 1 IBV type in a sample. In addition, the availability of microspheres with different spectral addresses makes it possible to extend the test to include more IBV types. Thus, it appears that this multiplex microsphere-based assay for IBV shows good potential as a research and a diagnostic tool.

Sources and manufacturers

a. Luminex xMAP system, Luminex Corp., Austin, TX.
b. Lasergene 8, DNASTAR Inc., Madison, WI.
c. Integrated DNA Technologies Inc., Coralville, IA.
d. High Pure RNA isolation kit, Roche Diagnostics Corp., Indianapolis, IN.
e. Titan One Tube RT-PCR System, Roche Diagnostics GmbH, Mannheim, Germany.
f. Recombinant RNase Inhibitor (5000 U), Takara Bio Inc., Otsu, Shiga, Japan.
g. DNA Engine Peltier Thermocycler, Bio-Rad Laboratories, Hercules, CA.
h. QIAquick gel extraction kit, Qiagen Inc., Valencia, CA.
i. Bio-Plex bead coupling protocol, Bio-Rad Laboratories, Hercules, CA.
j. Invitrogen Molecular Probes, Eugene, OR.
k. Bio-Plex microarray reader, Bio-Rad Laboratories, Hercules, CA.
l. Nanodrop spectrophotometer, Nanodrop Technologies Inc., Wilmington, DE.
m. Kindly provided by Dr. Holly S. Sellers, Poultry Diagnostic and Research Center, Athens, GA.
n. New England Biolabs Inc., Ipswich, MA.

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