Accurate Detection of Avian Respiratory Viruses by Use of Multiplex PCR-Based Luminex Suspension Microarray Assay

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A novel oligonucleotide suspension microarray (Luminex microsphere system) was developed for the rapid detection of avian respiratory viruses of major clinical importance. This test was optimized and validated with 70 clinical samples. The developed tool was accurate for high-throughput detection and differentiation of the most important avian respiratory viruses: avian influenza virus (AIV), Newcastle disease virus (NDV), infection bronchitis virus (IBV), and infectious laryngotracheitis virus (ILTV) in single- and mixed-virus infections. A multiplex reverse transcriptase PCR (RT-PCR), followed by a monoplex or a multiplex Luminex assays, were realized using a Luminex 200 analyzer instrument. The sensitivity, specificity, and reproducibility of the multiplex DNA suspension microarray system were evaluated. The results showed no significant differences in the median fluorescence intensity (MFI) value in monoplex and multiplex Luminex assays. The sensitivity and specificity proved to be completely concordant with monoplex real-time RT-PCR. We demonstrated that the multiplex DNA suspension microarray system is an accurate, high-throughput, and relatively simple method for the rapid detection of the main respiratory viruses of poultry.

Global broiler meat production has shown a steady increase and is expected to overtake pig meat production in 2020 (<http://www.fao.org> and <http://www.fas.usda.gov/>). Likewise, in Tunisia, the poultry sector is becoming an increasingly important agriculture sector, which is facing tough challenges due to infectious diseases in the region (1–4). Viral respiratory diseases are leading causes of economic losses in the poultry industry worldwide due to increased mortality, impaired growth, and reduced egg and meat production. The etiology of these diseases is complex and often involves more than one pathogen (5, 6). The most severe losses in terms of mortality are caused by very virulent viruses, mainly Newcastle disease virus (NDV) and avian influenza virus (AIV) (7–9). The two viruses give similar symptoms, ranging from a subclinical infection to a severe disease (10–12). Other respiratory viruses are also of major importance in terms of severity and impact on livestock production, mainly avian infectious bronchitis virus (IBV) and infectious laryngotracheitis virus (ILTV) (13). These viruses can cause disease independently, in association with each other, or in association with bacterial agents (6, 14). In addition, single- or multiple-virus infections may induce similar clinical signs/lesions, complicating diagnostic decisions and making it difficult for veterinarians to differentiate them clinically. Therefore, fast and sensitive detection techniques that are capable of differentiating between these different respiratory viral infections are needed for the surveillance of newly emerging viruses, outbreak management, as well as disease control.

In fact, accurate detection of respiratory avian disease-causing agents is an essential prerequisite for effective control. For this, a wide spectrum of methods is being developed and currently in use in diagnostic laboratories on a day-to-day basis. Although virus amplification combined with hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests are still considered to be the gold standard, they are highly time-consuming (7 to 14 days), labor-intensive, and have a low sample throughput (15). Many studies have demonstrated that molecular diagnostic assays, such as reverse transcriptase PCR (RT-PCR), show superior sensitivity

compared to that of conventional assays and are now becoming acceptable as new gold standards (16–18). In addition, real-time PCR in particular offers significant advantages due to its high sensitivity and rapid turnaround time (19–21). Most of the methods used for the detection of avian viral diseases are geared toward specific detection of a single target. Multiplex RT-PCR (mRT-PCR) assays involve simultaneous amplification of more than one infectious agent, using more than one primer pair. The advantage of such mRT-PCR is that it combines the sensitivity and rapidity of PCR and eliminates the need for testing clinical samples separately for each virus. These types of assays have already been used successfully for typing and subtyping influenza viruses (22–24) and diagnosing dual infections, such as NDV and AIV coinfection (25). Many other studies have also been performed using multiplex real-time RT-PCR to differentiate AIV, NDV, and IBV subtypes (26–39). To date, and to our knowledge, there are no reports describing simultaneous detection of NDV, AIV, IBV, and ILTV in a single sample.

The recent development of low-density suspension array technology, such as the Luminex xMAP technology, has offered an interesting alternative to multiplex real-time PCR. This format uses microbeads that are internally dyed with various proportions of red and infrared fluorescent dyes producing 100 distinct colors

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TABLE 1 Target genes, amplicon sizes, labels, and sequences of primers and probes used in the multiplex Luminex assay

Virus	Oligonucleotide	Primer/probe sequence (5'–3') ^a	Size (bp)	Target gene ^b	Reference
AIV	Fr_Phos	[Phos]AGATGAGTCTTCTAACCGAGGTCG	100	Matrix protein (M)	20
	Rv_Btn	[Btn]TGCAAAACATCTTCAAGTCTCTG			
	Probe	[AmC ₁₂]TCAGGCCCCCTCAAAGCCGA			
NDV	Fr_Phos	[Phos]AGTGATTGTCTCGGACCTTC	121	Polymerase (M) gene	19
	Rv_Btn	[Btn]CCTGAGGAGAGGCATTGCTA			
	Probe	[AmC ₁₂]TTCTCTAGCAGTGGGACAGCCTGC			
IBV	Fr_Btn	[Btn]GCTTTTGAGCCTAGCGTT	143	5' UTR	28
	Rv_Phos	[Phos]GCCATGTTGTCACTGTCTATTG			
	Probe	[AmC ₁₂]CACCACCAGAACCTGTCACCTC			
ILTV	Fr_Phos	[Phos]CCCCACCCAGTAGAGGAC	126	ICP4 gene	58
	Rv_Btn	[Btn]CGAGATACACGGAAGCTGATT			
	Probe	[AmC ₁₂]CAGTCTTTGGTCGATGACCCGC			

^a Phos, primer phosphorylated at the 5' end; Btn, primer labeled with biotin; AmC₁₂, Luminex probes synthesized with a 5'-amino modification and a 12-carbon methylene linker.

^b ICP4, infected cell protein 4.

(recently extended to 500) detected in a flow cytometry device (40). The Luminex assay has found increasing acceptance in both research and clinical laboratories for a variety of applications (41–52). It has already been developed for the detection and differentiation of many different viruses (43–45, 49, 53–56).

In the present study, we describe an established high-throughput assay facilitating reliable recognition of four avian respiratory viruses of major clinical importance. We have designed and validated, for the first time, a rapid identification method combining one-tube multiplex RT-PCR with Luminex xMAP bead hybridization and detection technology to simultaneously identify four clinically important avian respiratory viruses, NDV, AIV, IBV, and ILTV, in a single or a mixed infection.

MATERIALS AND METHODS

Virus strains and clinical materials. The AIV, NDV, IBV, and ILTV strains were obtained from the repositories of the National Veterinary Institute (SVA), Uppsala, Sweden. Clinical specimens from poultry verified by routine diagnostic procedures to be infected with respiratory viruses, including swabs (tracheal and cloacal) and internal organs (tracheal-lungs, liver-heart-spleen-kidney, and intestine-tonsil-cecal-cloaca), were also provided by the SVA.

Briefly, the virus strains used in the present study were characterized as follows. A virulent NDV (aPMV1/ch/Sweden/2008/G7b) strain was isolated from a laying hen flock showing dramatic loss of egg production in October 2008. The virus was isolated from the oviducts of diseased animals using specific-pathogen-free (SPF) embryonated hen's eggs. Samples showing hemagglutinating activity were subjected to a conventional HI test to specify the serotype. The virus was further characterized by complete genome sequencing (genotype 7b) and determination of the intracerebral pathogenicity index (ICPI = 1.8). The amino acid sequence motif of ¹¹²RRQRRF¹¹⁷ at the F protein cleavage site was indicative of a virulent pathotype of avian paramyxovirus-1 (aPMV-1). The IBV strain (M41-941125) was isolated during an outbreak investigation in 1994 from organ samples and tracheal swabs taken from a commercial layer flock experiencing drops in egg production, poor egg quality, and signs of respiratory disease. Virus isolation was performed according to the OIE recommendations, and the isolated virus was further characterized as strain M41 based on cross-neutralization tests with type-specific antisera and partial S1 gene sequencing. The AIV strain (A/Duck/Hungary/2/77 H5N2) was isolated from domestic ducks from the eastern part of Hungary in 1977 showing relatively severe respiratory signs. Subtyping of the isolated virus was determined by conventional HI and neuraminidase

inhibition (NI) tests, followed by its characterization by sequencing in 2008 (57). The virus belonged to the low-pathogenic (LP) Eurasian lineage of AIV based on the amino acid sequence of the proteolytic cleavage site of the hemagglutinin (HA) protein. The A489 ILTV strain was kindly provided by Walter Fuchs (Friedrich Loeffler Institute, Greifswald Insel Riems, Germany).

Viral nucleic acid extraction. Viral RNA was extracted from AIV, IBV, and NDV strains using TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Furthermore, total nucleic acids were also extracted from the ILTV strain and clinical samples by phenol-chloroform-isoamyl alcohol treatment and ethanol precipitation. The final extracted pellets were suspended in 20 µl of RNase-free water and stored at –20°C.

Design primers and oligonucleotide probes for real-time RT-PCR and Luminex assays. A high-throughput DNA suspension microarray assay was designed, allowing rapid and accurate detection and differentiation, in a single test, of the four avian respiratory viruses, AIV, NDV, IBV, and ILTV, in single or mixed infections. This quadruplex assay was adapted from previously published monoplex TaqMan real-time PCR assays targeting the matrix gene for AIV (20), the polymerase gene of NDV (19), the 5'-untranslated region of IBV (28), and the infected cell protein 4 (ICP4) gene of ILTV (58) (Table 1).

All primer and probe sequences were chosen after being thoroughly analyzed with the Premier Biosoft software and facilitated by applying the BLAST program for specificity prediction (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The Premier Biosoft software permitted the incorporation of existing proven primer and probe sequences together with new sequence targets to generate multiplexed assay designs. Available options for pre-design analyses, including melting temperature (T_m), G+C content, repeat run length, and variety of alignment rules were determined. Once the parameter settings are chosen, the software iteratively evaluates each multiplex component as it is added to the assay pool, minimizing the possibility of duplex and cross-reactivity issues. All Luminex probes were synthesized with a 5'-amino modification and a 12-carbon methylene linker (C₁₂) for bead conjugation (59). One primer was labeled with biotin (Btn), and the other was phosphorylated on the 5' end (44). The primers and probes are listed in Table 1.

Virus detection using monoplex real-time RT-PCR. Real-time RT-PCR primers and probes were adapted as previously described for AIV (20), NDV (19), IBV (28), and ILTV (58). The reaction volume was 15 µl, using 0.6 µl of primers (10 µM) and 0.2 µl of probe at 10 µM. A 2-µl volume of the template was added to the RT-PCR mixture. Real-time RT-PCR was performed on the Rotor-Gene Q system (Qiagen, Hilden, Germany) with a one-step RT-PCR (AgPath-ID one-step RT-PCR re-

agents; Applied Biosystems) at 45°C for 10 min, initial denaturation at 95°C for 10 min, followed by 40 PCR cycles of denaturation of 95°C for 10 s, and annealing and extension at 60°C for 45 s, with a single fluorescence acquisition step at the end of the annealing step. The cycle threshold (C_T) values from these assays represent relative quantification and broadly express the relative levels of viral nucleic acid. Samples exhibiting C_T values less than 40 were considered positive.

Multiplex RT-PCR for virus detection using Luminex xMAP. To confirm that individual primer pairs were specific enough to amplify the four different gene fragments, single-target PCR conditions, including primer concentration, annealing temperature, and DNA polymerase amount, were optimized. Thus, a uniform set of conditions were selected to perform multiplex PCR amplification, with the optimal annealing temperature being 60°C. After confirming that all individual primer pairs allowed amplification of each of the four target viral genomes in a single PCR, virus-specific primers were mixed together in a multiplex reaction. The multiplex one-step RT-PCR volume was 15 μ l, and the primer concentrations were 0.4 μ M. The PCR cycling profile was as follows: a reverse transcription step at 45°C for 10 min, an enzyme activation step at 94°C for 10 min, and finally, 40 PCR cycles with 10 s of denaturation at 95°C, 15 s of primer annealing at 60°C, and a 30-s extension at 72°C. All primer pair combinations were performed successfully and allowed amplification of all target viruses.

It has been shown that asymmetric PCR, which creates an excess of one of the strands, yields a better signal-to-noise ratio in Luminex equipment (53). In the present study, we have used Lambda exonuclease to digest the amplicon complementary to the target strand of the Luminex bead probe. The PCR products were digested using the following reaction mixture: the reaction volume was 15 μ l using 13.2 μ l of PCR product, 1.5 μ l of 10 $\times$ Lambda buffer, and 3 units of Lambda exonuclease (Fermentas Life Science, Burlington, Canada). The single-strand product was generated by incubating the PCR product at 37°C for 30 min, followed by an enzyme inactivation step of 15 min at 80°C.

Preparation of oligonucleotide probe beads. The probes contained a 5-terminal amino group with a seven-carbon spacer to allow covalent attachment to the microspheres (59). These probes were conjugated to color-coded beads (carboxylated microspheres; Luminex Corporation, Austin, TX) as follows. The beads (5×10^6 per oligonucleotide probe) were pelleted at $8,000 \times g$ for 2 min and resuspended in 50 μ l of 0.1 M 2-morpholinoethane sulfonic acid (MES) (pH 4.5), and aminated oligonucleotides (0.1 nmol) were added to the bead suspension. Following the addition of 25 μ g of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), the mixture was incubated at room temperature for 30 min in the dark. The addition of EDC and the incubation were repeated to ensure efficient conjugation. After the second 30-min incubation, the microspheres were washed by adding 0.5 ml of 0.02% Tween 20 and mixed gently by inverting the tube several times. The coupled beads were pelleted ($8,000 \times g$ for 2 min) and resuspended in 0.5 ml of 0.1% SDS by vortexing. Finally, the beads were pelleted again, resuspended to a concentration of 50,000 beads/ μ l in Tris-EDTA (TE [pH 8.0]) buffer, and stored protected from the light at 4°C.

Hybridization of biotinylated PCR amplicons to Luminex probes. The biotinylated PCR products were hybridized to the probe-coupled beads in round-bottom 96-well plates and then conjugated to streptavidin-phycoerythrin. The microsphere mixture consisted of four types of beads, with each one being coated with conjugated virus-specific probes (Table 1).

The bead stocks (50,000 beads/ μ l) were diluted in 1.5 $\times$ tetramethylammonium chloride (TMAC) hybridization buffer, such that 375 of each probe-coupled bead were present in each sample well (1,500 beads in total). The hybridization reactions were carried out in a total volume of 50 μ l/well consisting of 5 μ l of biotinylated PCR product, 13.5 μ l of TE buffer, and 31.5 μ l of the bead mixture in triplicate. For a background control, a no-template control (NTC) from the PCR assay was used.

According to previous studies (our unpublished data), the optimal

hybridization condition was incubation on a shaker plate at 55°C for 30 min at 600 rpm. After hybridization, the solutions were pelleted by centrifugation for 30 s. About 40 μ l of supernatant was carefully discarded without destroying the pellet to eliminate unbound PCR products. Then, the hybridized amplicons were fluorescently labeled by resuspending the pellet in 40 μ l of 1 $\times$ TMAC solution, containing a freshly prepared solution of streptavidin-R-phycoerythrin at a concentration of 0.01 mg/ml (Caltag Laboratories, Burlingame, CA, USA) and incubation in the dark at the same hybridization temperature for 30 min. Finally, the reaction plates were placed in a Luminex 200 instrument (Luminex Corporation, Austin, TX) at 55°C for bead enumeration and phycoerythrin fluorescence quantification. For each bead type, 100 microspheres were analyzed, representing 100 replicate measurements to calculate the MFI in each reaction (60). The signal-to-background ratio represents the MFI signals of positive controls versus the background fluorescence NTC. A positive signal was set as twice the MFI value of the NTC.

Sensitivity and multiplex detection of real-time RT-PCR and Luminex assays. Quantified viral nucleic acid materials were not available. However, since this study has utilized published and validated monoplex TaqMan assays conditions as the starting point for the development of the Luminex multiplex system, we can compare detection using the Luminex system with that of the corresponding TaqMan monoplex assays on identical dilutions of an unquantified viral nucleic acid preparation to get a useful estimate of the relative sensitivity. Thus, triplicate serial dilutions of unknown concentrations of the extracted DNA/RNA materials for each of the four virus strains were used for the direct sensitivity comparison of the new Luminex oligonucleotide suspension microarray, with monoplex real-time RT-PCR as gold standard assay. The detection limit was defined as the dilution containing the fewest copies of viral genome that still gave a positive result for all replicates.

Specificity and accuracy of the Luminex DNA suspension microarray. The specificity of the assay was theoretically assessed by evaluating the primers and probes for relevant homologies using the BLAST tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and assessing it experimentally by carrying out the multiplexed RT-PCR step against single- or multiple-virus DNA/RNA templates of the four avian viruses. In fact, the capability for multianalyte detection was investigated using several combinations of dilutions of the DNA/RNA standards of each of the four virus strains. The mixtures were then subjected to multiplex RT-PCR amplification and a subsequent Luminex array. Also, the individual probe beads were tested in the monoplex Luminex assay before pooling and testing them in a multiplex assay.

Interassay reproducibility. To evaluate the interassay reproducibility of the Luminex DNA suspension microarray, triplicates of one mixture of dilutions of the DNA/RNA standards of the four virus strains (10^{-6} AIV, 10^{-3} IBV, 10^{-4} NDV, and 10^{-5} ILTV) were tested in three independent reactions over separate days. The variability of the MFI values was assessed by analysis of variance.

Clinical validation of the Luminex DNA suspension assay. To validate the clinical applicability and accuracy of the Luminex assay, we have applied the assay on a set of 70 clinical samples from suspected poultry and compared the results with those of monoplex real-time RT-PCR as the gold standard assay.

Statistical analysis. Spearman's test is used to compute the correlation between the C_T of the monoplex real-time RT-PCR and MFI values of the multiplex Luminex assay. Some parameters considered were, for example, the P values, which typically should be less than 0.05 to validate the hypothesis in regard to the correlation, and the linear correlation coefficient ($-1 < \rho < 1$), which was calculated as such: if $\rho = 0$, there is no correlation between the two variables; and if $\rho = 1$ (or $\rho = -1$), it was concluded that the two variables are perfectly proportionally correlated (or inversely proportionally correlated, respectively). In addition, the coefficient of determination ($0 < R^2 < 1$) was used. Using this coefficient, we could judge and evaluate the regression between the two essays ($R^2 = 1$ for a good linearity and $R^2 = 0$ for no linearity).

TABLE 2 Comparison of the sensitivities of detection of AIV, NDV, IBV, and ILTV by the DNA suspension microarray and the monoplex real-time RT-PCR^a

Virus	Dilution	Detection of virus by Luminex assay		<i>C_T</i> for real-time RT-PCR ^b
		Multiplex	Monoplex	
AIV (A/Duck/Hungary/2/77 H5N2)	1 × 10 ⁰	+	+	11.49
	1 × 10 ⁻¹	+	+	15.07
	1 × 10 ⁻²	+	+	19.14
	1 × 10 ⁻³	+	+	22.41
	1 × 10 ⁻⁴	+	+	26.32
	1 × 10 ⁻⁵	+	+	30.45
	1 × 10 ⁻⁶	+	+	36.86
	7 × 10 ⁻⁷	–	–	–
	5 × 10 ⁻⁷	–	–	–
	3 × 10 ⁻⁷	–	–	–
	1 × 10 ⁻⁷	–	–	–
NDV (A/ch/Sweden/2008/G7d)	1 × 10 ⁰	+	+	10.67
	1 × 10 ⁻¹	+	+	13.37
	1 × 10 ⁻²	+	+	16.80
	1 × 10 ⁻³	+	+	19.86
	1 × 10 ⁻⁴	+	+	23.53
	1 × 10 ⁻⁵	+	+	26.69
	1 × 10 ⁻⁶	+	+	30.56
	7 × 10 ⁻⁷	+	+	34.84
	5 × 10 ⁻⁷	+	+	36.41
	3 × 10 ⁻⁷	+	+	37.93
	1 × 10 ⁻⁷	–	–	–
IBV (M41- 941125)	1 × 10 ⁰	+	+	12.41
	1 × 10 ⁻¹	+	+	15.86
	1 × 10 ⁻²	+	+	19.24
	1 × 10 ⁻³	+	+	23.33
	1 × 10 ⁻⁴	+	+	26.84
	1 × 10 ⁻⁵	+	+	29.57
	1 × 10 ⁻⁶	+	+	33.50
	7 × 10 ⁻⁷	+	+	36.95
	5 × 10 ⁻⁷	–	–	–
	3 × 10 ⁻⁷	–	–	–
	1 × 10 ⁻⁷	–	–	–
ILTV (A489)	1 × 10 ⁰	+	+	12.58
	1 × 10 ⁻¹	+	+	15.42
	1 × 10 ⁻²	+	+	19.19
	1 × 10 ⁻³	+	+	22.26
	1 × 10 ⁻⁴	+	+	25.75
	1 × 10 ⁻⁵	+	+	29.82
	1 × 10 ⁻⁶	+	+	34.18
	7 × 10 ⁻⁷	+	+	37.09
	5 × 10 ⁻⁷	–	–	–
	3 × 10 ⁻⁷	–	–	–
	1 × 10 ⁻⁷	–	–	–

^a Shaded rows represent the smallest dilution at which the Luminex assay detected the virus.^b A minus sign in this column means that the value is greater than 40 and considered negative.

Besides Spearman's test, the performances of the proposed method were also evaluated using the confusion matrix analysis. The confusion matrix contains information about the two assays. The following parameters were defined: true positive (TP) was the number of positive samples categorized as positive, false positive (FP) was the number of negative samples categorized as positive, false negative (FN) was the number of positive samples categorized as negative, and true negative (TN) was the number of negative samples categorized as negative. Based on these 4 metrics, the sensitivity ($S_n = TP/[TP + FN]$) and specificity ($S_p = TN/[TN + FP]$) of the novel multiplex Luminex assay could be evaluated.

RESULTS

Comparison of the sensitivity of the Luminex DNA microarray with the gold standard monoplex real-time RT-PCR. The sensitivity of the multiplex DNA microarray system was investigated and compared to the monoplex real-time RT-PCR as the gold standard. Serial dilutions of the extracted viral DNA/RNA of each one of the four virus strains were tested with both assays, and the results were compared, as shown in Table 2. For low concentrations of viruses, similar detection endpoints were obtained. The

FIG 1 Correlation between monoplex real-time RT-PCR cycle threshold (C_T) and multiplex Luminex assay mean fluorescence intensity (MFI) values for the detection of serial dilutions of AIV, NDV, IBV, and ILTV strains. Trendlines indicate statistically significant Spearman's correlation and good linearity (R^2 values of 0.97, 0.94, 0.85, and 0.97 for AIV, NDV, IBV, and ILTV, respectively).

Luminex assay could detect AIV, NDV, IBV, and ILTV at dilutions of 10^{-6} , 3×10^{-7} , 7×10^{-7} , and 7×10^{-7} , respectively (Table 2). The obtained sensitivity of the multiplex Luminex assay was equivalent to that shown for each viral targets detected by the monoplex assay. The raw MFI values were consistent when amplified in multiplex (Table 2). In addition, the multiplex DNA microarray assay could detect the same amount of each virus as in the monoplex real-time RT-PCR assay. Our results were analyzed by the Spearman's correlation coefficient, which was statistically significant for AIV, NDV, IBV, and ILTV ($P < 0.05$ for all; Spearman's ρ values of -0.98 , -0.97 , -0.92 , and -0.98 , respectively) (Fig. 1). Furthermore, negative Spearman's ρ values indicated that as C_T values increase, MFI values decrease, meaning that the two variables are inversely proportional.

Specificity and accuracy of the avian respiratory virus DNA suspension array. Prior to Luminex hybridization reaction, multiplex RT-PCR was carried out to amplify a single viral DNA/RNA template. The PCR amplicons with the expected lengths of 100, 121, 143, and 126 bp for the respective AIV, NDV, IBV, and ILTV genome fragments were obtained. No cross-reactivity and no unspecific amplifications were recognizable on agarose gels, as shown in Fig. 2.

Biotin-labeled PCR products of the various viruses tested were hybridized with the multianalyte bead-based suspension array. The tests showed that the multiplex Luminex hybridization is able to detect all targets with no positive cross-reaction signals (Fig. 3), which is quite compatible with the results obtained in the multiplex PCR. All four avian respiratory viruses were detected using the multiplex DNA suspension microarray with 100% specificity.

To evaluate whether the Luminex assay could accurately detect individual or multiple-virus DNA/RNA in a mixture, various dilutions of viral DNA/RNA of each of the four virus strains were mixed, subjected to multiplex RT-PCR amplification, and subsequently subjected to DNA suspension microarray hybridization.

Each mixture was tested in triplicate, and the data are shown as mean $\pm$ standard deviation (SD) MFI values (Table 3). The Luminex assay can still detect and differentiate each one of the four viruses present in the dilution mixtures (Table 3).

Reproducibility of the DNA suspension microarray. To evaluate the interassay reproducibility of the multiplex Luminex array, triplicates of one mixture of dilutions of the DNA/RNA standards of the four virus strains were tested in three independent reactions over separate days. The mean MFI values obtained represented with the SD demonstrate that reproducible results were achieved from the same combination of viruses when the assays

FIG 2 A representative agarose gel electrophoresis of multiplex RT-PCR products for the detection of AIV (A/Duck/Hungary/2/77 H5N2), NDV (A/ch/Sweden/2008/G7d), IBV (M41-941125), and ILTV (A489). Lane 1, 100-bp DNA size marker; lane 2, AIV product; lane 3, IBV product; lane 4, ILTV product; lane 5, NDV product; lane 6, negative control.

FIG 3 Representative data showing the comparison of the sensitivity of the monoplex and the multiplex Luminex assays for the detection of AIV (a), NDV (b), IBV (c), and ILTV (d).

were repeated 3 times (Fig. 4). Analysis of variance showed no significant difference in the MFI values of the triplicate mixtures, which were run over separate days ($P < 0.05$).

Performance of DNA microarray using field samples. To validate the performance of both assays, 70 field samples collected from poultry with suspected viral respiratory infections were analyzed by the DNA suspension microarray. These specimens were selected since they were already characterized by a routine monoplex real-time RT-PCR as the gold standard. Of the 70 samples that were tested, multiplex Luminex assay has successfully de-

tected a total of 57 positive avian respiratory virus samples, and 13 samples were negative for the four viruses. Of these, 11, 14, 22, and 10 specimens were positive for AIV, IBV, NDV, and ILTV, respectively. Two of these samples collected from poultry presented a coinfecting viruses. One chicken was coinfecting with both AIV and NDV, and the other was coinfecting with AIV and IBV (Table 4).

All samples identified by the Luminex assay proved to be 100% concordant with the results obtained by the monoplex real-time RT-PCR. In addition, a detailed confusion matrix analysis was

TABLE 3 Multiple-virus pathogen detection capability of the multiplex Luminex assay

Virus mixture	MFI (mean ± SD)			
	AIV	IBV	ILTV	NDV
10 ⁻¹ AIV + 10 ⁻¹ NDV	3,729.3 ± 218.7	25 ± 10.5	21.6 ± 10.7	1,623.3 ± 39.7
10 ⁻⁵ AIV + 10 ⁻⁵ NDV	1,326.7 ± 47.1	23 ± 4.3	17.6 ± 5.6	926.3 ± 45.4
10 ⁻⁶ AIV + 10 ⁻² IBV	344.6 ± 28.5	2,506 ± 46.5	20.3 ± 5.1	47.1 ± 1.4
10 ⁻³ AIV + 10 ⁻⁵ IBV + 10 ⁻⁴ NDV	2,885.2 ± 101.7	1,973.3 ± 36.9	25 ± 7.5	1,024.5 ± 16
10 ⁻³ NDV + 10 ⁻⁶ IBV	24.8 ± 4.2	1,205.7 ± 87.8	19.3 ± 9.6	46.2 ± 12.72
10 ⁻⁵ NDV + 10 ⁻⁵ IBV + 10 ⁻³ ILTV	28.5 ± 5.2	1,956.2 ± 124.5	1,155.8 ± 94.7	2,343 ± 88.4
10 ⁻⁴ AIV + 10 ⁻⁴ ILTV	1,734.7 ± 80.5	21.3 ± 1.1	1,026 ± 40	33.5 ± 3.1
10 ⁻⁵ IBV + 10 ⁻⁵ ILTV	40.6 ± 9.4	1,973.3 ± 36.9	732.6 ± 43.7	43.5 ± 6.3
10 ⁻³ AIV + 10 ⁻³ ILTV + 10 ⁻⁶ IBV	2,719 ± 102.3	1,205.7 ± 87.8	1,155.8 ± 94.7	12.6 ± 9.8
10 ⁻⁶ AIV + 10 ⁻³ IBV + 10 ⁻⁴ NDV + 10 ⁻⁵ ILTV	344.6 ± 28.5	2,343 ± 75	733.8 ± 47.4	1,024.5 ± 16

carried out to determinate the performance of the multiplex Luminex assay to detect and differentiate between the most important avian respiratory viruses compared to the monoplex real-time RT-PCR. The confusion matrix analysis indicated that the multiplex Luminex assay is 100% accurate in regard to its specificity and sensitivity (Table 4).

DISCUSSION

The rapid and accurate multiplex detection of avian respiratory viruses is potentially important in clinical diagnostic settings. The advantages of such specific and sensitive viral detection assays allow limiting the diagnostic cost of additional assays, as well as labor and instituting appropriate rapid disease control. Many methods have been developed and published as important tools for the identification of pathogens of the respiratory tract of poultry (16, 61–63). Most techniques tend to be either molecular (e.g., PCR and real-time PCR) or serological (e.g., enzyme-linked immunosorbent assay [ELISA]). Some of them are not suitable for

diagnoses, in some instances, to be used in multiplex assay. However, there is a lack of studies that discuss and investigate multiplex detections in the veterinary field (64), although, recently, nine avian respiratory pathogens were simultaneously detected based on GenomeLab gene expression profiler analyzer-multiplex PCR assay with 100% success as a rate of specificity (65). Most developed multiplex molecular assays have provided major steps to advance the diagnostic efficiency for the detection of human respiratory pathogens (17, 66–70). Various multiplex nucleic acid amplification and microarray-based methods have been evaluated for the simultaneous detection and identification of multiple human respiratory viruses (67, 71–73). Of these, the multianalyte suspension array is a rapid, sensitive, and high-throughput technique for viral detection compared to classical methods (53, 74, 75).

Multiplex PCR coupled with Luminex suspension arrays using microbeads for signal readout represents a recent diagnostic approach for clinical laboratories. To the best of our knowledge, the

FIG 4 Interassay reproducibility of the Luminex avian respiratory virus array results over three independent tests (a to c) using one mixture of dilutions of the DNA/RNA standards (10⁻⁶ AIV, 10⁻³ IBV, 10⁻⁴ NDV, and 10⁻⁵ ILTV). Red lines indicate the MFI mean values of triplicate experiments, and blue windows indicate the minimum and maximum MFI values, with SD shown as error bars.

TABLE 4 Sensitivity and the specificity of the multiplex Luminex assay for detecting viral DNA/RNA in 70 field samples

Target	Sensitivity		Specificity	
	TP/(TP + FN) ^a	%	TN/(TN + FP) ^a	%
AIV	11/11	100	59/59	100
IBV	14/14	100	56/56	100
NDV	22/22	100	48/48	100
ILTV	10/10	100	60/60	100

^a TP, true positive; FN, false negative; TN, true negative; FP, false positive.

Luminex technology has not been utilized before for the simultaneous detection of four clinically important avian respiratory viruses, which are AIV, NDV, IBV, and ILTV. We assessed the performance of the developed assay by comparing its sensitivity with the gold standard monoplex real-time RT-PCR and testing 70 avian field samples. The sensitivity of our test was the same as that of the monoplex real-time RT-PCR using serial dilutions of the extracted viral DNA/RNA of each one of the four virus strains. This is in agreement with previous observations (44). The limit of detection of the monoplex real-time RT-PCR in template copies per reaction was reported as 10³, 10³, and 10², respectively, for the AIV M gene, the NDV M gene, and the 5' untranslated region (UTR) for IBV (19, 20, 28). The multiplex assay is an efficient alternative to monoplex real-time PCR and greatly reduces the number of reactions required. However, multiplex real-time PCR is complicated by fluorescence receiver channel cross talk, which is not an issue with the Luminex technique that detects the fluorescent beads one by one in a flow cytometry device. The results demonstrated that the Luminex assay is specific for the target nucleic acid and easy to perform. The protocol could be made high throughput with a liquid handler and a 96-extracted-sample test in a rapid turnaround time of about 4 h (excluding DNA extraction). The approach is modular, and certain virus tests could be removed from the assay if the laboratory does not expect these avian viruses to be absent in the poultry flocks in the region. This assay can also be useful for research laboratories trying to understand the burden of avian respiratory viruses for poultry, which is worldwide related to mortality, impaired growth, reduced egg production or meat quality.

In summary, the DNA suspension array-based assay provides an alternative high-throughput molecular diagnostic platform for specific and sensitive detection of several major viruses commonly seen as important causes of viral respiratory diseases in poultry. The assay principle is straightforward, comprising few reaction steps in a single vessel. The run times are comparable to those of real-time PCR, with the benefit that the presence of several viruses can be analyzed in the same reaction. Although the reagent cost is higher than that for conventional PCR assays, it is reduced in proportion to the number of simultaneous analytes investigated. The clinical performance of the method was validated using a range of swabs as well as internal organs, and the potential utility of such a platform in veterinary diagnostics was demonstrated. This multiplex detection, using DNA suspension microarray, could provide more effective screening of viruses causing similar respiratory symptoms and in turn facilitates rapid counteractions, especially in case of outbreaks.

The establishment of this effective system for epidemiological surveillance will allow precise detection and identification of dif-

ferent avian respiratory viruses circulating in a region. This assay may close the gap of the absence of an efficient tool for multiplex diagnostic of these viruses and avoid the spread of infectious diseases in the poultry farms.

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