

Rapid Communication
Virology



Quantitative real-time PCR assays for the concurrent diagnosis of infectious laryngotracheitis virus, Newcastle disease virus and avian metapneumovirus in poultry

Jongseo Mo ¹, Michael Angelichio ², Lisa Gow ², Valerie Leathers ², Mark W. Jackwood ^{1,*}

¹Poultry Diagnostic and Research Center, Department of Population Health, College of Veterinary Medicine, University of Georgia, Athens, GA 30602, USA

²IDEXX Laboratories, Inc., Westbrook, ME 04092, USA

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*Corresponding author:

Mark W. Jackwood

Jackwood. Poultry Diagnostic and Research Center, Department of Population Health, College of Veterinary Medicine, University of Georgia, 953 College Station Road, Athens, GA 30602, USA.

Email: mjackwoo@uga.edu

<https://orcid.org/0000-0002-6777-5340>

ABSTRACT

Newcastle disease (ND), infectious laryngotracheitis (ILT) and avian metapneumovirus (aMPV) can be similar making it critical to quickly differentiate them. Herein, we adapted pre-existing molecular-based diagnostic assays for NDV and ILTV, and developed new assays for aMPV A and B, for use under synchronized thermocycling conditions. All assays performed equivalently with linearity over a 5 log₁₀ dynamic range, a reproducible (R² > 0.99) limit of detection of ≥ 10 target copies, and amplification efficiencies between 86.8%–98.2%. Using biological specimens for NDV and ILTV showed 100% specificity. Identical amplification conditions will simplify procedures for detection in diagnostic laboratories.

Keywords: Newcastle disease virus; avian infectious laryngotracheitis virus; avian metapneumovirus; quantitative real-time PCR

INTRODUCTION

Respiratory viral diseases in poultry can have similar clinical signs and are an important cause of economic loss in the industry. Infectious laryngotracheitis virus (ILTV), Newcastle disease virus (NDV) and avian metapneumovirus (aMPV) are viruses that frequently affect the respiratory tract of chickens and turkeys [1]. ILT is caused by *Gallid herpesvirus-1*, and is responsible for sporadic cases of acute respiratory disease in poultry around the world that can lead to trade embargoes [2]. NDV, which belongs to the family *Paramyxoviridae*, known as *Avian avulavirus-1* [3] and is further divided into class I and class II viruses. Class I viruses are commonly found in wild waterfowls, whereas the more diverse class II viruses are typically detected in a variety of birds in live bird markets, wild birds and poultry [4]. NDV infections have been confirmed in more than 200 different avian species, and like ILTV can also lead to trade embargoes [5]. aMPV, a member of the family *Pneumoviridae*, genus *Metapneumovirus* is the etiologic agent for swollen head syndrome in chickens [6] and severe rhinotracheitis in turkeys [7]. This virus is classified into four different subgroups (A, B, C, D), with subtype A and B mainly prevalent, especially in Europe [8,9].

ORCID iDs

Jongseo Mo
<https://orcid.org/0000-0003-1660-8646>
Michael Angelichio
<https://orcid.org/0000-0002-0909-7211>
Lisa Gow
<https://orcid.org/0000-0003-1594-0161>
Valerie Leathers
<https://orcid.org/0000-0003-0401-3581>
Mark W. Jackwood
<https://orcid.org/0000-0002-6777-5340>

Author Contributions

Conceptualization: Jackwood MW; Data curation: Mo J; Formal analysis: Mo J; Methodology: Mo J; Project administration: Jackwood MW; Validation: Mo J; Writing - original draft: Mo J; Writing - review & editing: Mo J, Jackwood MW, Angelichio M, Gow L, Leathers V.

Conflict of Interest

The authors declare no conflicts of interest.

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In our previous work, we evaluated quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) for detection and quantification of infectious bronchitis virus (IBV) types [10]. In this study, our objective was to build on that work by evaluating existing molecular tests for ILTV and NDV as well as develop and evaluate tests for aMPV-A and -B, all using the same thermocycling conditions. Using the same thermocycling conditions is important in a diagnostic laboratory setting so the same thermocycler can be used for the simultaneous detection of different pathogens.

MATERIALS AND METHODS

Design of primers, probes

Primers and probes for each test are listed in **Supplementary Table 1**. ILTV [11] and NDV [12] specific primer and probe sequences were selected from other literature, with aMPV-A and -B primer, probes newly designed for this study. The primer and probe sets targeted the gC gene of ILTV [11], M gene of NDV for generic detection, L gene of class I NDV [12] and the N gene of aMPV-A and -B. The purpose of including the L gene of low virulence class I NDV as a target was to broaden detection of NDV. Primers and probe specificity for aMPV was verified by an in-depth *in silico* examination using the BLAST search tool at NCBI (www.ncbi.nlm.nih.gov; USA).

A set of primers and probe targeting endogenous avian RNA for use as an internal positive control (IPC) were also designed for the aMPV-A and -B, and NDV assays. For the ILTV assay, another set of primers and probe targeting a DNA-IPC was also designed. The DNA-IPC was a synthesized template that was added to the sample mix prior to nucleic acid extraction.

Preparation of synthetic DNA standards

Synthetic DNA templates mimicking the biological target sequence can be utilized for high-quality standardization of assay performance [13]. Double-stranded synthetic DNA standards were designed and synthesized (Integrated DNA Technologies, USA) using the consensus target sequence for each assay. The number of copies for each DNA standard was determined by the manufacturer. The DNA templates were serially diluted 10-fold and used to calculate analytical sensitivity and limit of detection (LOD). The 5 μ L of each diluted template was added per reaction.

An IPC, was designed and included to rule out potential false negative results [14]. A unique set of PCR primers and probe targeting endogenous avian RNA for use in avian originated samples was added to the NDV and aMPV assays. As biological avian samples were not used in DNA template standardization step, a separate DNA-IPC template mimicking the target sequence of the specific endogenous avian RNA was synthesized and spiked into the reaction mix. The IPC primers and probes, along with the DNA-IPC template were also added into the ILTV assay reaction mix. The gene and sequence information of the IPCs is proprietary.

The sample mix preparation for each PCR assay is described in detail in **Supplementary Table 2**. Each assay included positive and negative controls. The NDV primer and probe sets for both generic NDV-M gene and class I L gene detection were run in separate reactions as well as together, and assay performance was compared. Each assay was tested a minimum of 3 times and all samples were run in triplicate.

Preparation and processing of clinical and biological samples

Thirty class II NDV and 30 ILTV-positive specimens and 30 previously tested negative samples were prepared and processed to validate the qRT-PCR assays. All available samples used in this study were from our laboratory archives. Clinical and biological samples positive for class I low virulence NDV, aMPV-A and -B were unavailable. The clinical and biological sample nucleic acid extraction and master mix preparation are described in **Supplementary Table 2**. Each assay included positive and negative controls.

Thermocycling conditions for qRT-PCR

Amplification and detection were conducted in an Applied Biosystems 7500 fast Real-Time PCR system (Applied Biosystems, USA) under the following conditions, which were different from previously published conditions for NDV [12] and ILTV [11]: RT at 50°C for 15 min and denaturation at 95°C for 1 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 30 sec with optics on. Test duration was 81 min, and thermocycling conditions for all assays were identical. Samples were always run in triplicate to test for reproducibility. Although ILTV is a DNA virus, to synchronize thermocycling conditions with other assays, the RT incubation step was included.

RESULTS

Analytical sensitivity qRT-PCR assays using synthetic DNA standards

The specificity of the primers and probe for each assay was examined *in silico* and it was found that they were specific for their intended usage (data not shown). The dynamic range of all assays spanned 5 log₁₀ units from 10 to 10⁵ copies per reaction with a slope ranging from -3.37 to -3.68 and R² values ≥ 0.99, at a LOD of ≥ 10 copy numbers and with amplifications efficiencies of 86.8%–98.2% (**Supplementary Table 3, Fig. 1**). The assays detected 10 copy numbers in all triplicate runs. The mean Ct values, slopes of the standard curve and R² values are shown in **Supplementary Table 3**. The artificially added IPC templates were successfully amplified and did not appear to interfere with the amplification of the target template (data not shown). Likewise, the primer and probe set for NDV-M and L in the same sample mix did not appear to interfere with amplifying their target template (data not shown).

Validation of virus-specific assays using clinical and biological specimens

The validation results of NDV-M and ILTV assays using clinical specimens are presented in **Table 1**. Both assays detected their target virus in 100% of known positive samples, and none of the negative samples or non-target avian respiratory viruses were detected indicating high specificity. The endogenous avian RNA-IPCs in the NDV assay and the artificially spiked DNA-IPCs in the ILTV assay were successfully co-amplified and did not show any interference regarding performance (data not shown).

The aMPV-A and -B assays were tested against other viruses and no cross reactivity was observed (data not shown). In the absence of biological specimens for the aMPV-A and -B assays, *in silico* PCR analysis was carried out by the FastPCR software [15] and reported in **Supplementary Table 4**. The analysis predicted that the assays could successfully generate amplicons from the intended aMPV strains, and no cross-detection was indicated with the non-target aMPV types, suggesting high specificity of the assays.

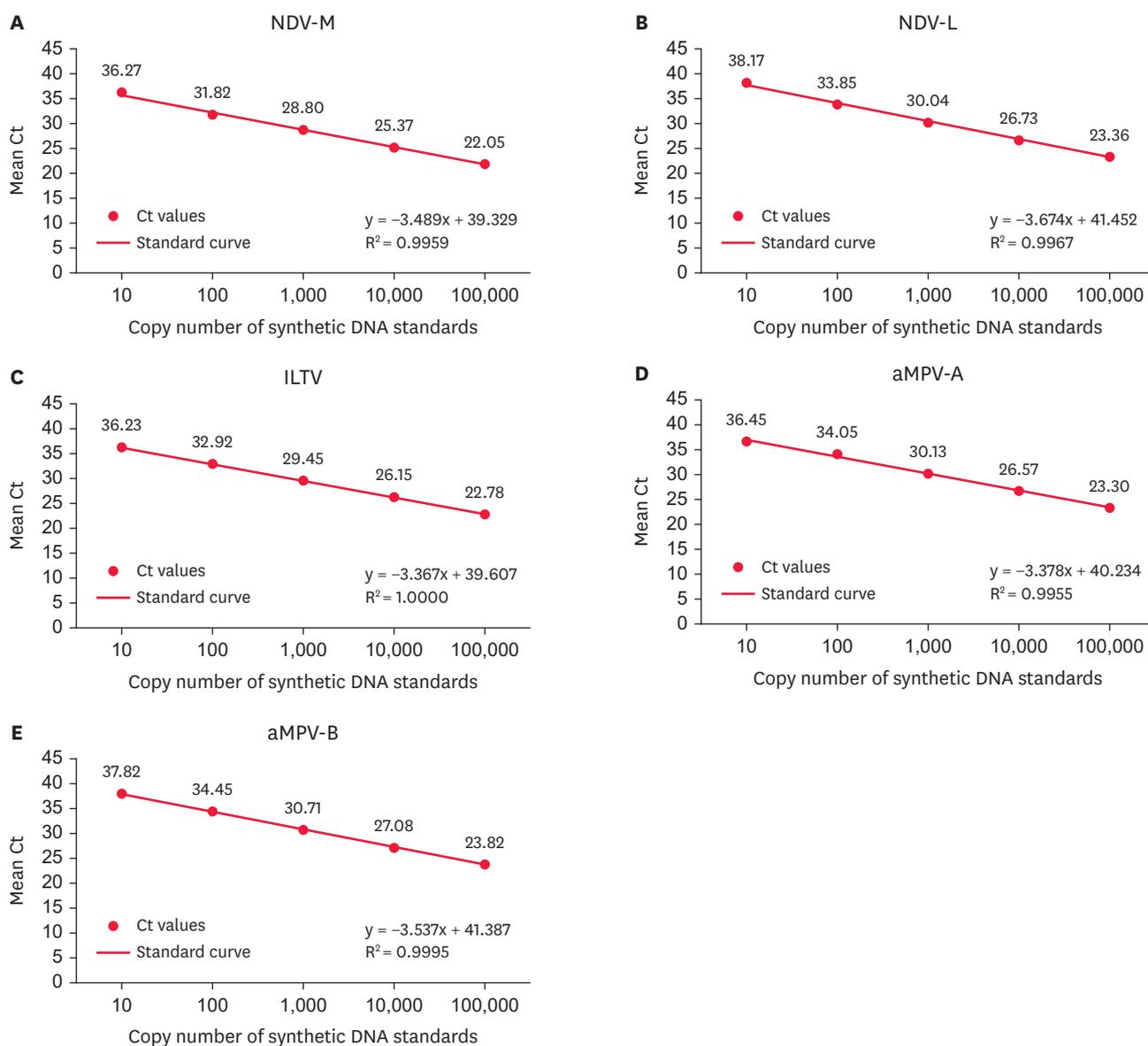


Fig. 1. Analytical sensitivity of qRT-PCR assays. Standard curves for (A) NDV-M, (B) NDV-L, (C) ILTV, (D) aMPV-A, (E) aMPV-B assays presenting the mean Ct plotted against the relative input copy numbers (\log_{10}) of synthetic DNA standards that were tested in triplicate. Synthetic DNA standards were serially diluted by 10-fold at a 5 \log_{10} range, starting from 10^5 copies down to 10 copies per reaction. qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; NDV, Newcastle disease virus; ILTV, infectious laryngotracheitis virus; aMPV, avian metapneumovirus.

Table 1. Sensitivity, specificity, PPV and NPV of NDV-M, ILTV qRT-PCR assays using 30 true positive and 30 true negative* clinical and biological samples

Assay type	Sensitivity	Specificity	PPV	NPV	Viruses†		
					NDV	ILTV	Neg/IBV
NDV-M	100	100	1.00 (100)	1.00 (100)	30/0	0/30	0/30
ILTV	100	100	1.00 (100)	1.00 (100)	0/30	30/0	0/30

Values are presented as % or number (%).

PPV, positive predictive value; NPV, negative predictive value; NDV, Newcastle disease virus; ILTV, infectious laryngotracheitis virus; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; IBV, infectious bronchitis virus; Neg, negative.

*Negative samples that did not contain the target virus, were previously confirmed by the Poultry Diagnostic and Research Center's diagnostic laboratory (University of Georgia, USA) using real-time PCR tests and consisted of swabs and biological samples. Samples containing IBV were also used for negative samples. Positive samples for NDV consisted of 30 choanal swabs, whereas 20 tracheal swabs and 10 biological conjunctiva-associated lymphoid tissue samples were used for ILTV.

†Number of samples testing positive/Number of samples testing negative.

DISCUSSION

Respiratory diseases are common and can be similar in poultry and diseases caused by NDV, ILTV and aMPV can cause significant economic losses [1]. Therefore, it is crucial to rapidly detect and differentiate these viruses so that countermeasures can be implemented. Building on our previous work detecting IBV types by qRT-PCR, we synchronized thermocycling parameters for previously developed ILTV and NDV assays as well as newly developed aMPV assays to establish a concurrent testing process. Synthetic DNA templates were utilized for standardization and verification of the assays, as they hold advantages for that type of analysis [13]. Although RNA templates are appropriate for determining the efficiency of RT-PCR assays, which can be quite variable, our goal was to standardize and verify the assays based on analytical sensitivity and LOD, making defined copies of synthetic DNA template more appropriate. All assays performed equivalently, with an LOD of 10 copies. The NDV and ILTV assays from this study demonstrated a comparable or even higher sensitivity to the originally described NDV (M gene LOD: 10^3 copies, L gene LOD: 10^2 copies) [12] and ILTV (LOD: 10^2 copies) [11] assay conditions. The average efficiencies of the assays were within the acceptable range of 80%–115% [16], and above (≥ 0.99) the generally recommended $R^2 \geq 0.95$ for standard slopes between -3.0 and -3.9 . The unnecessary RT step in the ILTV assay, to synchronize thermocycling conditions with other assays, did not affect its performance (**Supplementary Table 3, Fig. 1**).

Using clinical and biological samples, 100% of the known positive samples were determined as positive by the NDV-M and ILTV assays (**Table 1**). The endogenous avian RNA-IPC in NDV and aMPV-A and -B assays and the artificially spiked DNA-IPC in ILTV assays did not appear to interfere with performance. Multiplexing of these assays wasn't possible due to a decrease of sensitivity and efficiency (data not shown). In this study we were not able to test the aMPV assays and the NDV-L assay with clinical or biological samples. However, the assays were capable of detecting synthetic templates with high sensitivity and specificity, and *in silico* analysis predicted that the assays could generate amplicons from the aMPV strains successfully, with no non-target aMPV type cross-detection. Similar to previous reports, this indicates that the assays will likely give reliable results when applied to biological samples [13].

The provision of these assays, which all use the same thermocycling conditions, will facilitate field diagnostics in terms of accuracy and functional simplicity.

SUPPLEMENTARY MATERIALS

Supplementary Table 1

Primers and probe* used in this study

[Click here to view](#)

Supplementary Table 2

The sample mix preparation for each real-time PCR assay

[Click here to view](#)

Supplementary Table 3

Efficiency of NDV-M/L, ILTV, aMPV-A, aMPV-B qRT-PCR assays

[Click here to view](#)**Supplementary Table 4***In silico* PCR prediction of aMPV-A/B assays[Click here to view](#)**REFERENCES**

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