Virology 515 (2018) 29–37

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/virology

A multiplex real-time RT-PCR for simultaneous detection of four most common avian respiratory viruses

Nacira Laamiri[a,b,*], Rim Aouini[a,b], Boutheina Marnissi[a], Abdeljelil Ghram[a], Issam Hmila[a]

[a] University Tunis El Manar, Institut Pasteur de Tunis, Laboratory of Epidemiology and Veterinary Microbiology, 13 Place Pasteur, 1002 Tunis-Belvedere, Tunisia.
[b] University of Carthage, Faculty of Sciences Bizerte, 7021 Zarzouna Bizerte, Tunisia

ARTICLE INFO

Keywords:
AIV
Avian respiratory viruses
IBV
ILTV
Multiplex
NDV
Real-time RT-PCR

ABSTRACT

A one-step multiplex real-time reverse transcription-PCR (rRT-PCR) assay was developed for simultaneous detection and quantification of four avian respiratory viruses: avian influenza virus (AIV), infectious bronchitis virus (IBV), Newcastle disease virus (NDV) and infectious laryngotracheitis virus (ILTV). In comparison with the singleplex rRT-PCR, the specificity, the sensitivity and the reproducibility of the new assay were evaluated and validated using 70 clinical samples. The optimal cutoff point, the corresponding limit of quantification (LoQ) and the limit of detection (LoD) were statistical established based on receiver operating characteristic (ROC) curve analysis. The results showed that the multiplex assay presents higher sensitivity and specificity. Correlation coefficients (R^2) and amplification efficiencies (E) of all singleplex and multiplex rRT-PCR reactions are within the acceptable range. The 95% LoDs of multiplex assay were in the range [3–19] copies genomic/ µl, and its corresponding cutoff cycles were in the range [34.16–36.59]. No competitive inhibition for the detection of the four targets and no specific amplification or cross reactivity with other tested viruses was observed. Excellent results were attained in the inter-assay and intra-assay reproducibility evaluation. All identified samples by the multiplex rRT-PCR assay proved to be 100% concordant with the results of the singleplex assays. The results achieved showed that the multiplex assay is very suitable as a routine laboratory test for rapid and specific detection and quantification of co-infections in field samples.

1. Introduction

As the development of polymerase chain reaction (PCR) technology has rapidly progressed, the need for sophisticated assays for molecular diagnosis has also accepted as the new gold standard for detecting nucleic acids in samples of various origins. Likewise, the PCR has become a routine tool in many research laboratories to monitor a wide variety of templates across a range of scientific specialties (Espy et al., 2006; Mackay, 2004; Mackay et al., 2002). Among these different PCR techniques, real-time PCR (rPCR) has engendered wider acceptance for its improved rapidity, higher specificity, sensitivity, reproducibility and reduced risk of carrying-over contaminations. One of the most interesting aspects of rPCR, based on detection of fluorophoric labeled oligonucleotides, such as Taqman®, is its possibility to detect conveniently multiple targets in the same PCR reaction (multiplex PCR) (Elnifro et al., 2000). Ideally, a multiplex rPCR should be able to detect differentiate and provide quantitative results for many different targets without any cross reaction.

The rPCR method has become a main tool for laboratory diagnosis and presents a key for surveillance and control of infectious diseases (Binnicker, 2015; Hoffmann et al., 2009; Loginov et al., 2016; Rewar et al., 2015; Stoute et al., 2016; Uehara-Ichiki et al., 2013). Furthermore, diagnostic of infectious diseases would greatly benefit from the possibility of simultaneous detection of multiple targets. For instance, the etiology of avian viral respiratory diseases is complex and often involves more than one pathogen infecting the respiratory tract (Ahmed et al., 2009; Malik et al., 2004). As such, avian influenza virus (AIV), infectious bronchitis virus (IBV), Newcastle disease virus (NDV) and infectious laryngotracheitis virus (ILTV) are the most common respiratory viruses among poultry farms that cause huge economic losses each year (Achenbach and Bowen, 2011; Alexander, 2000; Cavanagh, 2003; Sakai et al., 2006; Wang et al., 2008). These avian respiratory viruses can bring disease independently, in association with each other, or in association with bacterial agents (Malik et al., 2004; Wang et al., 2008; Ali and Reynolds, 2000). Besides, single or multiple avian viral infections may produce alike clinical signs. Therefore, it is too difficult to distinguish these various etiologic agents based on clinical signs and symptoms, which make accurate and timely laboratory detection of

* Corresponding author at:
E-mail address: naciralaamiri@yahoo.fr (N. Laamiri).

https://doi.org/10.1016/j.virol.2017.11.021
Received 9 August 2017; Received in revised form 26 November 2017; Accepted 27 November 2017

Table 1
Primers and probes used in this work.

Virus	Oligo	Primer/probe sequence (5′ – 3′)	Genomic region	Amplicon Size (bp)	Target gene	Reference
AIV	Fr	AGATGAGTCTTCTAACCGAGGTCG	25–48	100	Matrix protein (M)	(Spackman et al., 2002)
	Rv	TGCAAAAACATCTTCAAGTCTCTG	101–124			
	Probe	TCAGGCCCCCTCAAAGCCGA	64–83			
NDV	Fr	AGTGATTGTCTCGGACCTTC	4100–4120	121	Polymerase (M) gene	(Wise et al., 2004)
	Rv	CCTGAGGAGAGGCATTTGCTA	4199–4220			
	Probe	TTCTCTAGCAGTGGGACAGCCTGC	4169–4193			
IBV	Fr	GCTTTTGAGCCTAGCGTT	391–408	143	5′ untranslated region (UTR)	(Callison et al., 2006)
	Rv	GCCATGTTGTCACTGTCTATTG	512–533			
	Probe	CACCACCAGAACCTGTCACCTC	473–494			
ILTV	Fr	CCCCACCCAGTAGAGGAC	143906–143923	126	Infected cell protein 4 (ICP4) gene	(Ou et al., 2012)
	Rv	CGAGATACACGGAAGCTGATTT	144010–144031			
	Probe	CAGTCTTTGGTCGATGACCCGC	143949–143971			

viruses important for early diagnostic of avian respiratory diseases. However, the complex composition presents a challenge for detecting and quantifying the viruses. As accurate identification of involved pathogens may influence the disease management, fast and reliable diagnosis of the causative agent is highly desirable.

Quite a lot of such rPCR method for the detection of avian viruses has been published providing useful references for people working on the diagnostic of avian respiratory viral infections (Stoute et al., 2016; Acevedo et al., 2013; Dormitorio et al., 2013; Fraga et al., 2016; Hurtado et al., 2016; Jang et al., 2011; Kis et al., 2013; Lee et al., 2016; Thanh et al., 2010). Unfortunately, most of these rPCR assays cover limited virus strains or apply under different cycling conditions. Therefore, a reliable comprehensive one-step multiplex real-time reverse transcription-PCR (rRT-PCR) assay, covering all important pathogens (suitable for multiplex screening or specific quantitative identification with fast turnaround time and identical cycling parameters), is urgently needed, allowing simultaneous and effective detection of unknown samples.

In this work, we propose an effective one-step rRT-PCR assay for multiplex detection of the most important avian respiratory viruses, independently or combined, within clinical samples. The efficiency of the proposed procedure, formulated by the one-step multiplex rRT-PCR assay based on four singleplex reactions, is compared to a quadruplex format in which all viruses are detected in a single reaction. All assays were optimized at a universal thermal cycling condition and evaluated under singleplex and multiplex conditions for the detection and quantification of total nucleotide acid. They were proved to be reliable a molecular tool for early diagnostic and consequently mean to address threat of viral respiratory diseases.

2. Materials and methods

2.1. Virus strains and clinical samples

For specificity and sensitivity testing, as well as evaluation of the multiplex assay, different panels of clinical samples and reference materials were used in this study.

The specificity of the multiplex assay were tested with the following reference strains: AIV isolate strain (A/chicken/Tunisia/145/2012(H9N2)), H3N2 influenza virus strain (A/Switzerland/9715293/2013), H1N1 influenza virus strain (A/Puerto Rico /8/1934), influenza B virus strain (B/Brisbane/60/2008), IBV local isolates strain (Tunisia/335/2001 and Tunisia/20/2000) (Bourogâa et al., 2009), ILTV Tunisian isolate strain (Kaboudi et al., 2016), H120 IBV vaccine strain, NDV LaSota vaccine strain, avian metapneumovirus vaccine strain (VCO3/60616, B subtype), live IBDV (Infectious Bursal Disease virus) intermediate vaccine strain and attenuated strain 1133 of avian reovirus.

A total of 70 clinical samples from suspected poultry, including tracheal and cloacal swabs received for routine diagnosis or surveillance program between 2014 and 2015, is used to evaluate the multiplex one-step rRT-PCR assay performances and its assess diagnostic effectiveness.

2.2. Viral nucleic acid extraction

Viral DNA and RNA were co-extracted from reference strains and clinical samples using Viral Gene-spin™ Viral DNA/RNA Extraction Kit, according to the manufacturer's directions (iNtRON Biotechnology). One hundred fifty microliters of Dulbecco's Modified Eagle's Medium (Gibco™ DMEM) suspensions of reference strains, tracheal and cloacal swabs and internal organs as described below, were used in the extraction phase. To avoid contamination, standard precautions were considered. Nucleic acids were eluted in a final volume of 50 µl and either analyzed directly or stored at −80 °C until further use.

2.3. Primer and hybridization probe set design

For specific detection of single or mixed infections of AIV, NDV, IBV and ILTV, primer and probe sequences were adapted from previously published singleplex Taqman® rRT-PCR systems targeting the matrix gene for AIV (Spackman et al., 2002), the polymerase gene of NDV (Wise et al., 2004), the 5′-untranslated region of IBV (Callison et al., 2006), and the infected cell protein 4 (ICP4) gene of ILTV (Ou et al., 2012) (Table 1). These assays were shown to be sensitive and specific to detect all strains.

Different published primers and probes of singleplex Taqman® rRT-PCR assays specific for AIV, NDV, IBV and ILTV viruses were tested to determine whether they could be used together in a multiplex assay. Potential primer and probe sequences were selected after being thoroughly analyzing the Premier Biosoft software and the BLAST program (http://blast.ncbi.nlm.nih.gov /Blast.cgi). BLAST analyses were performed to check the specificity of the primers and the probes against other closely related genome sequences. The Premier Biosoft software allowed the combination of existing proven primer and probe sequences together with new sequence targets to yield multiplexed assay designs and to choose identical cycling parameters. The primer pairs and probes specific to each virus were grouped in a multiplex reaction on the basis of the following criteria: internal primer binding properties for hairpin and primer-dimer potential, length of the desired amplicon, G-C content, and melting temperatures (Tm) of the probes and primers. When the parameter settings are defined, Premier Biosoft software assesses each multiplex component as it is added to the assay pool, avoiding the risk of cross reactivity issues.

The primers and Taqman® probes were obtained from Invitrogen™ (Thermo Fisher Scientific). Taqman® hydrolysis probes were labeled at the 5′-end with the reporter dyes and quenched with Blackhole Quencher™ 1 or 2 (BHQ 1 or 2) at the 3′-end. The singleplex methods described for AIV, IBV, NDV and ILTV detection were used as baseline controls for the development of a multiplex format assay. For more convenience, the method was somewhat modified and each Taqman®