Detection of infectious bronchitis virus and Avian metapneumovirus by a duplex semi-nested reverse transcription–polymerase chain reaction assay

Detecção do vírus da bronquite infecciosa das galinhas e do metapneumovírus aviário utilizando uma reação de transcrição reversa com reação em cadeia pela polimerase duplex

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Abstract

A duplex RT-PCR assay is reported for the simultaneous detection of avian infectious bronchitis virus (IBV) and avian metapneumovirus (aMPV), the causative agents of major diseases in poultry. The duplex RT-PCR assay optimized showed a detection limit of 10^{-3} (10^{1} EID_{50}/50µ L for IBV and $10^{0.5}$ EID_{50}/50µ L for aMPV, respectively when two viruses were mixed and 10^{-1} for each one separated (10^{3} EID_{50}/50µ L for IBV and $10^{2.5}$ EID_{50}/50µ L for aMPV, respectively. It was specific, sensitive and applicable for the rapid detection of these viruses in clinical samples.

Keywords: IBV. aMPV. PCR. Diagnosis.

Resumo

Descreve-se um ensaio de duplex RT-PCR assay para a detecção simultânea do vírus da bronquite infecciosa das galinhas (IBV) e do metapneumovirus aviário (aMPV), agentes etiológicos de doenças de elevada importância em avicultura. A duplex RT-PCR otimizada mostrou um limiar de detecção de 10^{-3} (10^{1} EID_{50}/50µ L para IBV e $10^{0.5}$ EID_{50}/50µ L para aMPV, respectivamente, quando da combinação dos dois vírus e 10^{-1} para cada um dos vírus em separado(10^{3} EID_{50}/50µ L para IBV e $10^{2.5}$ EID_{50}/50µ L para aMPV, respectivamente. O ensaio foi demonstrado como específico, sensível e aplicável à rápida detecção destes vírus em amostras clínicas.

Palavras-chave: IBV. aMPV. PCR. Diagnostic.

Introduction

3Avian metapneumovirus (aMPV) belongs to subfamily *Pneumovirinae* within the *Paramyxoviridae*[5]. IBV and/or aMPV not only replicate inthe respiratory but also in the reproductive tract, affecting the quality of eggs and causing a range of reproductive tract abnormalities, including peritonitis and ovary and oviduct atrophy[6,7,8].

Methods such as multiplex RT-PCR assays allow for the simultaneous detection of several pathogens, thereby optimizing the use of reagents and decreasing personnel time[9].The advantage of these assays is that they combine the sensitivity and quickness of the PCR and eliminate the necessity of evaluating clinical samples separately for each virus; these assays have been used usefully for typing and sub-typing of viruses such as IBV, avian influenza[10,11,12] and for multiple detection of Newcastle disease virus (NDV), avian influenza virus and aMPV[13].

This article reports on the preliminary results of the optimization and evaluation of a duplex semi-nested reverse transcription PCR (dsnRT-PCR) assay for the simultaneous and differential detection of IBV and aMPV in different organs of poultry.

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Material and Method

A total of 45 samples (9 pools) of enteric contents, lungs, trachea, kidneys, and reproductive tractspools from multiple ages broiler and layers of poultry farms from São Paulo State, Brazil with clinical signs of infection by IBV and aMPV (reproductive and /or respiratory problems) were collected as organ-specific pools of five birds after necropsy. The samples were prepared as 20 % w/v suspensions in DEPC-treated water and clarified at 5,000 x g for 15 min; the supernatants were then collected for virus detection.

Virus reference strains used to optimize the dsnRT-PCR assay wereMassachusetts H-120 of IBV and aMPV subtype Avaccine strains withtiters of 10^4 EID_{50}/50µ L and $10^{3.5}$ EID_{50}/50µ L, respectively. To the evaluation of the specificity of dsnRT-PCR, vaccine strains of NDV (strain La Sota) and Infectious bursal disease virus IBDV (strain D78) were used. IBV and aMPV reference strains were serially10-fold diluted in DEPC-treated water for the analytical sensitivity assay.

Total RNA was extracted with TRIzol reagent™ (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions, directly from tissue suspensions (for IBV and aMPV screening), negative (ultrapure water) and positive (IBV H120 and aMPV subtypes A and B) controls and NDV and IBDV.

Complementary DNA (cDNA) was synthesized using Moloney-Murine leukemia virus reverse transcriptase™ (M-MLV) (Invitrogen, Carlsbad, CA) in a 20-µL final reaction volume.

Briefly, 3.5 µL of RNA were denatured at 95°C and added to the reverse transcription mix containing 1x First Strand Buffer™ (Invitrogen, Carlsbad, CA), 1 mM of each dNTP, 10 mM of DTT, 1.25 ng/µL of random hexamers (Invitrogen, Carlsbad, CA) and 100U of M-MLV. The reaction was then carried out at 42°C for 60 minutes.

For the PCR and semi-nested steps, previously published primers were used targeting the 3'UTR for the detection of IBV[14]and the G gene for the detection of both subtypes A and B of aMPV, with amplicons size of size 179bp for IBV (primers UTR41+ ATGTCTATCGCCAGGGAAATGTC, UTR 31- GGGCGTCCAAGTGCTGTACCC and UTR11- GCTCTAACTCTATACTAGCCTA) and 444bp for aMPV (primersG1+ GGGACAAGTATC (T/C) (C/A) (T/G) AT and G6-CTGACAAATTGGTCCTGATT). A serial titration of reagents was performed to optimize the dsnRT-PCR protocol, including concentration of reagents (DNA polymerase and $MgCl_2$) and primer efficiency and the assay was finally optimized as follows:

For PCR (first-round amplification), the reaction mix was prepared in a volume of 50 µL with 5 µL of cDNA, 1x PCR Buffer ™ (Invitrogen, Carlsbad, CA), 0.2 mM dNTP, 0.5 µM of each primer (UTR 41+, UTR 11-, G6- and G1+), 1.5 mM $MgCl_2$, 1.25 U Platinium Taq DNA Polymerase ™ (Invitrogen, Carlsbad, CA) and DEPC-treated water to complete 50µL. The PCR reaction was carried out under the following conditions in a thermal cycler (Eppendorf Mastercycler ™): 35 cycles of denaturation at 94°C for 1 min, annealing at 48°C for 1.5 min., elongation at 72°C for 2 min and 1 cycle of 10 min at 72°C.

For the second-round amplification (semi-nested PCR in the case of IBV and re-amplification in the case of aMPV), the reaction mix was prepared in a volume of 50 µL comprised of 5 µL of first round PCR product, 1x PCR Buffer ™ (Invitrogen, Carlsbad, CA), 0.2 mM dNTP, 0.5 µM of each primer (UTR 41+, UTR 31-, G6- and G1+), 1.5 mM $MgCl_2$, 1.25 U Platinum Taq DNA Polymerase ™ (Invitrogen, Carlsbad, CA) and DEPC-treated water to complete 50µL. The second-round amplification was carried out with the same conditions described for the first-round amplification.

Amplicons were visualized under UV light after electrophoresis in 1.5% agarose gel in 0.5 x TBE buffer

followed by staining with ethidium bromide (0.5µg/mL). For estimating product length, 100 bp DNA ladder was used as a marker.

Each step (RNA extraction reverse transcription-polymerase chain reaction, nested reactions and electrophoresis) was carried out in separate rooms in order to avoid amplicon carry over.

The analytical sensitivity of the dsnRT-PCR assay was evaluated by testing sequential 10-fold dilutions in DEPC-treated water (ranging from 10^{-1} to 10^{-6}) of IBV and aMPV vaccine stock solutions. The viruses were tested both as volume/volume combinations and in separate. The RNA was extracted and cDNA was synthesized from each dilution as described above.

Besides, the detection limit was measured separately for IBV and aMPV by adding cDNA prepared with random primers for IBV and aMPV controls to the multiplex mix separately and combined (10^{-1}-10^{-6} dilutions) using the specific primers in separate reactions and the other conditions were as described above.

The analytical specificity of the dsnRT-PCR was assessed using RNA extracted from NDV and IBDV, processed and tested as described above for the cDNA synthesis, first round and nested steps of the dns RT-PCR.

Results and Discussion

The dsnRT-PCR assay optimized showed a detection limit of 10^{-3}(10^{1} EID_{50}/50µ L for IBV and $10^{0.5}EID_{50}$/50µ L for aMPV, respectively) when two viruses were mixed and 10^{-1} for each one separated (10^{3} EID_{50}/50µ L for IBV and $10^{2.5}$ EID_{50}/50µ L for aMPV, respectively). Thus, when two viruses were mixed, better results were obtained, possibly because of a carrier RNA effect when a higher amount o RNA is available in an ethanol-based precipitation step of RNA extraction.

Furthermore, the dsnRT-PCR was found more sensitive than each simple (primer-specific) RT-PCR for each virus. The sensitivity of simple RT-PCR for IBV was $10^{-2}$and 10^{-1} for aMPV. The analytical specificity of the dsnRT- PCR assay was supported by the observation that only IBV and aMPV were detected whilst NDV and IBDV were not.

A comparison between IBV the assay detection limit form the present study and that described by the authors of the article from which the 3UTR primers were taken was not possible as the authors do not provide detection limits in their study[15].

To determine the quality of the assay and the competence of the dsnRT-PCR test performance in clinical samples, 45 samples (9 pools of five birds) of enteric contents, lungs, trachea, kidneys, and reproductive tracts pools from multiple ages broiler and layers with clinical signs of infection by IBV and aMPV (reproductive and /or respiratory problems) were evaluated and all of them were found positive to IBV by the dsnRT-PCR, while none was positive for aMPV.

The use of RT-PCR for detection of avian pathogens is not new and different strategies to detect individual viruses have been reported[16], but the detection of IBV and aMPV in a same reaction has not employed.

It was not the aim of the present investigation to apply this test to a large amount of clinical samples in a field study, as this will be the issue of future publications.

Nonetheless, the results presented herein indicate the utility of this assay in detecting these two viruses in samples of birds to be implemented in any molecular biology laboratory.

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