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Detection of Newcastle disease virus using nucleic acid sequence-based amplification

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Abstract

Newcastle disease (ND) is a contagious and widespread avian disease affecting most species of birds. ND virus (NDV) is the only member of the avian paramyxovirus serotype 1 (APMV1) causing ND outbreak in bird flocks. The technique of nucleic acid sequence-based amplification (NASBA) is a potential method to rapidly and reliably detect NDV isolates. Here, we describe an effective and unprecedented method for detecting NDV strains of all pathotypes. A conserved region of the fusion protein gene was used for designing oligonucleotides specific to all NDV pathotypes. The dynamic range of this NDV NASBA detection method is comparable to virus culture and therefore the NDV NASBA method is a potential alternative for NDV screening and surveillance.

Keywords: Newcastle disease; NASBA; NDV; RNA amplicons; Surveillance; Disease screening

1. Introduction

Newcastle disease (ND), a contagious and widespread avian disease affecting most species of birds, is one of the most serious diseases of poultry caused by ND virus (NDV). NDV is the only member of the avian paramyxovirus serotype 1 (APMV1) [1]. Viruses of the genus *Rubulavirus* and the family *Paramyxoviridae* have a negative-sense, single-stranded RNA genome consisting of approximately 15 kb. The genome encodes six major polypeptides in the 5′-to-3′ direction, including RNA-directed RNA polymerase, hemagglutinin-neuraminidase, fusion protein, matrix protein, phosphoprotein, and nucleoprotein [2]. Nine serotypes (PMV-1 to PMV-9) exist based upon the species or the type of bird the virus was isolated from and the location of isolation [3].

NDV strains were generally classified into the highly pathogenic (velogenic), intermediate or moderately pathogenic (mesogenic), and lowly pathogenic (lentogenic) pathotypes. However, pathotype groupings are not necessarily clear-cut and a response to mild strains may be exacerbated by other conditions, such as pre-existing infections and environmental factors. Furthermore, ND infections in birds vary widely regardless of virulence exhibited in chickens, diagnosing ND in various birds poses a challenge to disease control and timely isolation of infected flocks.

Currently, the preferred method of diagnosis is virus isolation and subsequent characterization of the agent [4], the latter

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of which includes pathogenicity tests and serological tests. However, the virus isolation and characterization processes require a relatively longer period of time (in terms of days) [5], therefore rapid routine detection of NDV is not so feasible when massive sample size is encountered in a situation of outbreak. Recently, a real-time reverse-transcription PCR (RRT-PCR) assay system has been developed as a molecular diagnostic technique to rapidly screen NDV [6]. This RRT-PCR assay showed promising specificity to NDV isolates of various pathotypes and sensitivity of detecting as few as 10 infectious particles, however, the authors cautioned that RRT-PCR cannot replace virus isolation completely on an individual sample basis and that molecular diagnostic technique should be useful for rapid screening and surveillance of at-risk poultry flocks [6].

As an alternative to PCR-based molecular diagnostic technique, nucleic acid sequence-based amplification (NASBA) method is an RNA-based amplification technique with a potential to rapidly and reliably detect NDV isolates. NASBA is a transcription-based amplification system especially suitable for the detection of RNA-based targets [7]. NASBA allows continuous amplification of nucleic acids in a single mixture at a single temperature (isothermal condition) [8]. The amplification step involves an enzyme mixture containing avian myeloblastosis reverse transcriptase (AMV-RT), ribonuclease-H, and bacteriophage T7 DNA-dependent RNA polymerase, and two specially designed target-specific DNA oligonucleotide primers, capable of achieving amplification of as much as 10^9-fold [8]. A third target-specific capture probe allows detection of specific RNA amplicons among background of non-specific amplification products. Recent utilization of fluorescent molecular beacon probes in the NASBA reaction has allowed the development of real-time NASBA to detect bacterial or viral pathogens [9–11].

In this paper, we describe an effective and unprecedented method for detecting NDV strains of all pathotypes, using a series of experiments to demonstrate the proof of principle of the NDV NASBA method. The objective of this NASBA method is to allow accelerated detection of NDV strains that are potentially applicable to rapid screening and disease monitoring.

2. Materials and methods

2.1. NDV strains

NDV strains selected for this study were isolated between August 2001 and October 2002 in the northeast part of China (Table 1). These isolates originated during outbreaks of ND in chicken and pigeon flocks representing at least six flocks of birds. The chicken flocks involved had been vaccinated with an NDV vaccine, LaSota/46, at least once depending on the age when the ND outbreaks occurred. Systematic collection of NDV samples from the outbreak area allowed epidemiological study of prevalent NDV strains to monitor efficacy of vaccine use on chicken flocks and to allow development of molecular diagnostic methods for rapid screening of NDV. The isolates were designated according to the origin and year of isolation, Ji Lin (JL) and Hei Long Jiang (HLJ). PPMV1/pigeon/China/HLJ-4/02 was isolated from a racing-pigeon flock that suffered an outbreak of ND with 50.1% mortality rate shortly after an international race in 2002.

2.2. Design of NASBA primers and capture probe

Design of NASBA primers and capture probe was based on the F gene. More than 500 NDV F gene sequences were aligned by Clustal X software and a conserved region was used for the oligonucleotide design using a commercially available software (Primer Express 2.0, Applied Biosystems, USA). The resultant oligonucleotides specific to NDV F gene conserved region were analyzed by the BLAST sequence comparison algorithm (http://www.ncbi.nlm.nih.gov/BLAST).

2.3. RNA preparation and NASBA amplification reaction

RNA was extracted using a silica-based Isolation Reagent Kit (bioMérieux bv, Boxtel, Netherlands) modified from a previously published method [12]. Extracted RNA samples were used immediately after extraction or stored at −80 °C until use. RNA amplification by NASBA technique was performed as previously described ([13,14]). Briefly, RNA sample (5 μl) was added to amplification mixture (10 μl) according to the protocol of the Amplification Basic Kit (bioMérieux), followed by incubation at 65 °C for 5 min, and cooling to 41 °C for 5 min. Once cooled, enzyme mix (5 μl containing RNase-H, T7 RNA polymerase, and AMV-RT) was added and the reaction mixture was incubated for 90 min at 41 °C to allow isothermal amplification of RNA amplicons. Detection reagents were prepared by vortex mixing a suspension comprising biotinylated NDV-specific oligonucleotide capture probe bound to streptavidin-coated paramagnetic beads until an opaque solution was formed. The bead-oligo suspension (10 μl) and 10 μl ruthenium-labelled electrochemiluminescent (ECL) probe were mixed. Twenty microlitres of this mixture was added to 5 μl of the NASBA product and incubated for 30 min at 41 °C. Finally, 0.3 ml of Assay Buffer (bioMérieux) was added to the hybridization tube and the ECL reading was performed in the NucliSens ECL Reader (bioMérieux) according to the manufacturer's protocol.

2.4. Virus isolation and characterization

Viruses were isolated by two or three passages using embryonated, specific-pathogen-free (SPF) eggs. Pathotyping of the isolates initially involved virus inoculation of 10-day-old, embryonated, SPF eggs to determine the mean death time (MDT) of the embryos. Further virus characterization included inoculation of 1-day-old SPF chicks to determine the intracerebral pathogenicity index (ICPI), hemagglutination (HA) and hemagglutination inhibition tests, which were performed according to standard procedures described previously ([4,5]).

Table 1
Details of selected NDV isolates used in this study

Sample	Strain	Origin	Host	GenBank number	Genotype
JL-1	APMV1/chicken/China/JL-1/02	JL	Chicken	AY208680	VII
JL-2	APMV1/chicken/China/JL-2/02	JL	Chicken	AY208681	VII
JL-3	APMV1/chicken/China/JL-3/02	JL	Chicken	AY208682	VII
JL-4	APMV1/chicken/China/JL-4/02	JL	Chicken	AY208683	VII
JL-5	APMV1/chicken/China/JL-5/02	JL	Chicken	AY208684	VII
JL-6	APMV1/chicken/China/JL-6/02	JL	Chicken	AY208685	VII
JL-7	APMV1/chicken/China/JL-7/02	JL	Chicken	AY208686	VII
JL-8	APMV1/chicken/China/JL-8/02	JL	Chicken	AY208687	VII
JL-9	APMV1/chicken/China/JL-9/02	JL	Chicken	AY208688	VII
JL-10	APMV1/chicken/China/JL-10/02	JL	Chicken	AY208689	VII
JL-11	APMV1/chicken/China/JL-11/02	JL	Chicken	AY208690	VII
JL-12	APMV1/chicken/China/JL-12/02	JL	Chicken	AY208691	I
JL-13	APMV1/chicken/China/JL-13/02	JL	Chicken	AY208692	VII
JL-14	APMV1/chicken/China/JL-14/02	JL	Chicken	AY208693	VI
HLJ-1	APMV1/chicken/China/HLJ-1/02	HLJ	Chicken	AY208694	VII
HLJ-2	APMV1/chicken/China/HLJ-2/02	HLJ	Chicken	AY208695	VII
HLJ-3	APMV1/chicken/China/HLJ-3/02	HLJ	Chicken	AY208696	I
HLJ-4	PPMV1/pigeon/China/HLJ-4/02	HLJ	Pigeon	AY208697	VI
HLJ-5	APMV1/chicken/China/HLJ-5/02	HLJ	Chicken	AY208698	VII
HLJ-6	PPMV1/pigeon/China/HLJ-6/02	HLJ	Pigeon	N/A	VI

2.5. Phylogenetic analysis

To assess the genetic lineage among the NDV strains, phylogenetic analysis was performed with 3′ portions of 72 fusion protein (F) gene sequences (47–420 nt), 48 F gene subsequences (329–582 nt), and the entire coding region of the 26 F gene sequences (47–1708 nt). The NDV strains used in this phylogenetic analysis are listed in Tables 1 and 2. Sequence alignment and phylogenetic distance of F gene were performed with the Clustal V method using DNAStar software (MegAlign, version 1.03). This method used a multiple-alignment algorithm and the unweighted-pair group method

Table 2
Information of NDV strains from GenBank

GenBank number	Strain	Origin	Genotype	GenBank number	Strains	Origin	Genotype
AY028995	CH-A7/96	China	VII	AF458015	ZhJ-3/97	China	VI
AF364835	Ch/98-3	China	VII	AF456439	JS/2/98/Go[a]	China	VI
AF358786	TW/2000	Taiwan	VII	AF400615	NDV-JL-2/97	China	VI
AF164966	Ow/Tw/2209/95	Taiwan	VII	AF400616	NDV-BJ-3/97	China	VI
U62620	Taiwan95	Taiwan	VII	AF401999	Qingdao/SD/1/97	China	VI
AF162714	GPMY/QY97-1	China	VII	M24692	D26/76	Japan	I
AF140343	LZ-NDV	Unknown	VII	AF217084	Queensland (V4)	Australia	I
AF456437	GD/1/98/Go[a]	China	VII	D00243	Ulster2C/67	North Ireland	I
AF456440	JS/4/01/Go[a]	China	VII	Y18898	Clone 30	Unknown	II
AF456442	JS/5/01/Go[a]	China	VII	AF077761	LaSota/46	USA	II
AF456443	JS/9/01/Go[a]	China	VII	AF375823	B1	Japan	II
AF456444	JS/7/01/Go[a]	China	VII	AF534997	ZJ/2000	China	II
AF431744	ZJ1	China	VII	AF079323	DB5	China	II
AF458010	JS-3/00	China	VII	AF079324	PNDV1	Unknown	II
AF458011	XJ-2/97	China	VII	AF397009	SDBZ-S98	China	II
AF458012	FJ-2/99	China	VII	AF400614	NDV-JL-1/97	China	II
AF458013	JS-2/98	China	VII	M21881	Aus-Victoria/32	Australia	III
AF458014	JX-2/99	China	VII	M18456	Miyadera/51	USA	IV
AF458016	ZhJ-2/86	China	VI	M24702	HER/33	UK	IV
AF458017	Sh-2/98	China	VI	M33855	Texas	USA	IV
AF458018	Sh-1/97	China	VI	AF048763	AF2240	Malaysia	VIII
AF458019	XJ-3/97	China	VI	AF079172	$F_{48}E_9$	China	IX
AF458020	XJ-1/91	China	VI	AF458009	FJ-1/85	China	IX
AF458021	JX-1/94	China	VI	AF458023	ZhJ-1/85	China	IX
AF458022	JS-1/97	China	VI	AF456435	JS/1/97/Go	China	IX
AF358785	Ch/98-1	China	VI	AF079322	DB3	China	IX
AF358787	Ch/99	China	VI				

[a] "Go" denotes goose as the host.

with an arithmetic mean algorithm to derive a preliminary phylogeny. The final phylogenetic tree (dendrogram) was generated by the neighbor-joining method. The 100 replications were bootstrapped to construct a consensus phylogenetic tree.

2.6. Analytical sensitivity and specificity

Analytical sensitivity of the NDV NASBA system was compared with the viral culture method. An ND viral stock (APMV1/chicken/China/JL-9/02) of known ICPI was serially diluted 10-fold from 10^{-8} to 10^{-1}. The serial dilution was prepared from the viral stock obtained by two independent culture experiments. Aliquots (100 μl) of each dilution were subjected to viral culture or NASBA detection method according to the procedures described in the previous subsections. For analytical specificity, viral culture was performed on samples collected from ND outbreaks in China to identify NDV strains. The collected virus from NDV culture positive samples was lysed in the Lysis Buffer provided in the Isolation Reagent Kit (bioMérieux bv, Boxtel, Netherlands). RNA samples were extracted from the viral lysate samples and were subsequently tested by the NDV NASBA method. Human and avian influenza samples exhibiting similar hemagglutination activity were also subjected to the NDV NASBA method.

3. Results and discussion

3.1. NASBA primer and capture probe sequences

Primers and capture probe used in the NDV NASBA amplification were based on a conserved region of the F gene after over 500 NDV F gene sequences were aligned and analyzed. All designed oligonucleotides were searched against the GenBank sequence depository by BLAST and results demonstrated matches to a wide variety of ND viral isolates. The silico analysis shows that these oligonucleotides do not exhibit significant match with other non-NDV sequences. The sequences of the primers and capture probe used in the NDV NASBA method were as follows: ECL-ND-FP: GATGCAAG GTCGCATATGAGAGCTT(G/A)ATCAC(C/T/A)GG(T/C)(A/T)ACCCTAT(T/A/C)CT; T7-ND-FP: AATTCTAATACGACT CACTATAGGGAGAAGGAGGCA(A/T)A(T/C)CC(C/T)TTG GTTGT(G/A); and ND-CP: TGCGTGC(T/C)ACCTA(C/T)TT GGA(G/A)ACCTT(G/A)TCTG.

3.2. Phylogenetic analysis of NDV F gene

In order to investigate the genetic lineage among the NASBA-tested NDV strains with other NDV strains found in Taiwan, Japan, Australia, Europe, and USA, phylogenetic analysis was carried out using the NDV F gene sequences. In total, F gene sequences of 72 NDV strains (shown in Tables 1 and 2) were aligned and phylogenetic relationship among these 72 strains is depicted by the phylogenetic dendrogram (Fig. 1). NDV genotypes I–IX, except V, are represented in the dendrogram, which indicates genetic distances among the studied NDV strains. In fact, it can be observed that ND outbreaks in China during the period of 2001 and 2002 were predominantly caused by NDV genotype VII, although outbreaks caused by genotypes I and VI also occurred previously. Analysis of phylogenetic relatedness among NDV strains isolated from various ND outbreaks allows the appropriate selection of ND vaccines of the correct genotypes to prevent further ND outbreak.

3.3. Analytical sensitivity of NDV NASBA

NDV strain stocks (APMV1/chicken/China/JL-9/02), cultured by two independent experiments, with known ICPI were serially diluted 10-fold from 10^{-8} to 10^{-1}. Equal volume (100 μl) of diluted virus was assayed by viral culture or the NDV NASBA detection method. For one batch of serial dilution samples, the NDV NASBA method could detect down to the 10^{-6} dilution, whereas viral culture down to 10^{-5} only. For the other batch of serial dilution samples, the NDV NASBA method could detect down to 10^{-8} dilution, whereas viral culture down to 10^{-7} only. This may suggest that the NDV NASBA method supersedes viral culture slightly in sensitivity. But comparison of the analytical sensitivity between NDV NASBA and viral culture will be further verified using more experimental replicates of serially diluted NDV viral stocks. The NDV NASBA method offers the advantage of expedited assay speed as the entire NASBA experiment can be finished as quickly as 5 h.

3.4. Analytical specificity of NASBA

Selected NDV strains with determined HA values were used in the NASBA assay, and result was tabulated in Table 3. All tested NDV isolates were predominantly genotypes VII and I, which all gave rise to positive NASBA ECL readings. (Samples are considered positive when NASBA ECL readings are higher than a cut-off value of 5000.) These tested NDV strains represent genetic lineage covering a wide range in the dendrogram (Fig. 1). Regarding non-specificity, the NDV NASBA oligonucleotides did not show any cross hybridization with other viruses exhibiting similar hemagglutination activity (e.g. human and avian influenza viruses, Table 3). The detection of NDV strains shown in Table 3 indicates the feasibility of using the NASBA method in screening for the presence of NDV nucleic acid in collected samples.

Nucleic acid sequence-based amplification with electrochemiluminescent (NASBA/ECL) detection is a continuous isothermal reaction, which will result in amplification of RNA to one billion fold, while PCR can only amplify DNA greater than one million fold [8]. The isothermal condition used in NASBA can minimize the unexpected hybridization properties of primers resulting from cyclic changes of temperature as seen in PCR. The isothermal condition, in fact, allows easier standardization of the assaying condition using an equipment as simple as a water bath. The NDV NASBA method described here provides a potentially rapid and robust approach for the detection of NDV isolates

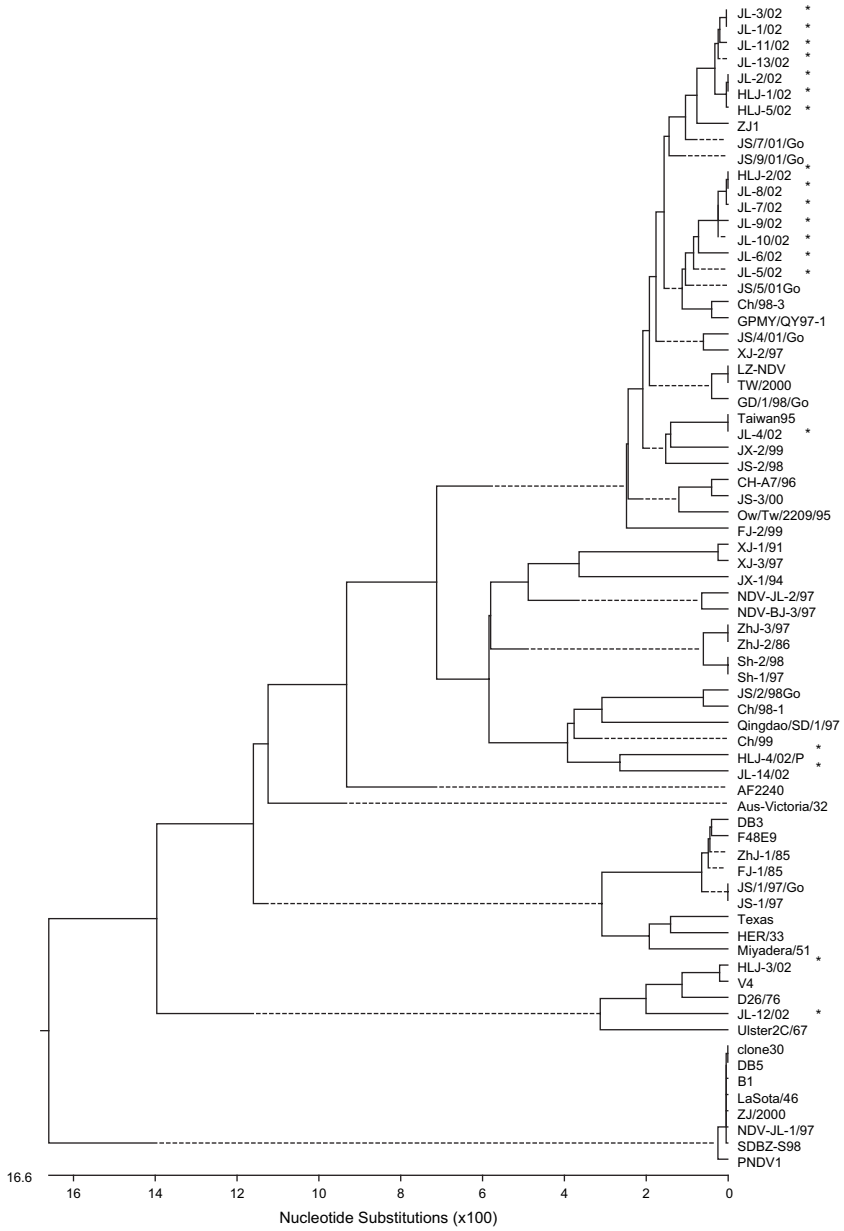

Fig. 1. Phylogenetic tree of NDV strains based on nucleotide sequences from a portion (338–592 nt) of the F gene. Asterisks denote strains tested by the NDV NASBA detection method as shown in Table 1.

in veterinary samples. Using this assay system, reliable identification of the ND viral strains can be made within 5 h. Without strand separation (or denaturation) of DNA, the NASBA assay can amplify specific sequences of single-stranded RNA targets amongst genomic DNA contaminants [7]. We have previously demonstrated the suitability of the NASBA/ECL method in detecting RNA-based viruses, such as avian influenza virus [13] and foot-and-mouth disease virus [14]. To allow the NASBA assay to be more portable for use in places where actual field samples are being tested, NASBA amplification coupled with an enzyme-linked nucleic acid capture (ENAC) detection assay has been developed for the detection of NDV. The magnitude of amplicon detection for NASBA/ECL and NASBA/ENAC is equivalent (unpublished data), thus enabling NASBA users to avoid the cost of purchasing an ECL detector, which at times may be relatively expensive.

In summary, a molecular diagnostic technique based on NASBA to detect ND viral isolates has been developed. The NDV NASBA assaying system has slightly higher sensitivity than viral culture and offers the advantage of rapidly generating results within a few hours. The assay, although requiring further evaluation with large number of field samples, has the potential to be a rapid alternative to serological tests for routine screening of poultry and other birds to help to minimize disease outbreak.

Table 3
Analytical specificity of the NDV NASBA detection

Sample	Strain (genotype)	ECL reading	Result	HA value
JL-12	APMV1/chicken/China/JL-12/02 (I)	2,083,168	Positive	2^7
JL-1	APMV1/chicken/China/JL-1/02 (VII)	2,001,483	Positive	2^7
JL-3	APMV1/chicken/China/JL-3/02 (VII)	10,000,001	Positive	2^6
JL-4	APMV1/chicken/China/JL-4/02 (VII)	1,816,032	Positive	2^6
JL-5	APMV1/chicken/China/JL-5/02 (VII)	10,000,001	Positive	2^6
JL-6	APMV1/chicken/China/JL-6/02 (VII)	1,920,858	Positive	2^6
HLJ-6	PPMV1/pigeon/China/HLJ-6/02 (VI)	431,781	Positive	2^6
JL-7	APMV1/chicken/China/JL-7/02 (VII)	2,069,820	Positive	2^8
JL-8	APMV1/chicken/China/JL-8/02 (VII)	1,829,415	Positive	2^7
JL-9	APMV1/chicken/China/JL-9/02 (VII)	10,000,001	Positive	2^7
Human influenza A 1	NA	632	Negative	NA
Human influenza A 2	NA	379	Negative	NA
Avian influenza H5 subtype	NA	3188	Negative	NA
Negative control	NA	650	Negative	NA

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References

[1] Alexander DJ. Newcastle disease and avian paramyxovirus infections. In: Calnek BW, Barnes HJ, Beard CW, McDougald LR, editors. Diseases of poultry. 10th ed. Ames, Iowa: Iowa State University Press; 1997. p. 541–69.

[2] Millar NS, Emmerson PT. Molecular cloning and nucleotide sequencing of Newcastle disease virus. In: Alexander DJ, editor. Newcastle disease virus. Boston, MA: Kluwer Academic Publishers; 1988. p. 79–97.

[3] Alexander DJ. Newcastle disease and other avian paramyxoviruses. Reviews in Science and Technology 2000;19:443–62.

[4] Office International des Epizootes (OIE). Manual of standards for diagnostic tests and vaccines. 5th ed. Paris, France: Office of International des Epizootes; 2004.

[5] Alexander DJ. Newcastle disease. In: Purchase HG, Arp LH, Domermuth CH, Pearson JE, editors. A laboratory manual for the isolation and identification of avian pathogens. 3rd ed. Kennett Square, PA: American Association of Avian Pathologists, Inc.; 1989. p. 114–20.

[6] Wise MG, Suarez DL, Seal BS, Pedersen JC, Senne DA, King DJ, et al. Development of a real-time reverse-transcription PCR for detection of Newcastle disease virus RNA in clinical samples. Journal of Clinical Microbiology 2004;42:329–38.

[7] Deiman B, van Aarle P, Sillekens P. Characteristics and application of nucleic acid sequence-based amplification (NASBA). Molecular Biotechnology 2002;20:163–79.

[8] Compton J. Nucleic acid sequence-based amplification. Nature 1991;350:91–2.

[9] de Baar MP, van Dooren MW, de Rooij E, Bakker M, van Gemen B, Goudsmit J, et al. Single rapid real-time monitored isothermal RNA amplification assay for quantification of human immunodeficiency virus type I isolates from groups M, N, and O. Journal of Clinical Microbiology 2001;39:1378–84.

[10] Keightley MC, Sillekens P, Schippers W, Rinaldo C, St. George K. Real-time NASBA detection of SARS-associated coronavirus and comparison with real-time reverse-transcription-PCR. Journal of Medical Virology 2005;77:602–8.

[11] Schneider P, Wolters L, Schoone G, Schallig H, Sillekens P, Hermsen R, et al. Real-time nucleic acid sequence-based amplification is more convenient than real-time PCR for quantification of *Plasmodium falciparum*. Journal of Clinical Microbiology 2005;43:402–5.

[12] Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-Van Dillen PM, van der Noordaa J. Rapid and simple method for purification of nucleic acids. Journal of Clinical Microbiology 1990;28:495–503.

[13] Collins RA, Ko LS, So KL, Ellis T, Lau LT, Yu AC. Detection of highly pathogenic and low pathogenic avian influenza subtype H5 (Eurasian lineage) using NASBA. Journal of Virological Methods 2002;102:213–25.

[14] Collins RA, Ko LS, Fung KY, Lau LT, Xing J, Yu AC. A method to detect major serotypes of foot-and-mouth disease virus. Biochemical and Biophysical Research Communications 2002;297:267–74.