

Original Article

Development of RT-PCR Using External and Internal Positive Controls Based on 5' Untranslated Region (UTR) for Molecular Detection of Avian Infectious Bronchitis Virus

Mahdi A¹, Ghalyanchi-Langeroudi A^{2*}, Hosseini SM¹, Soleimani M³, Mohseni AH⁴

1. Department of Microbiology, Faculty of Biological Sciences, University of Shahid Beheshti, Tehran, Iran.
2. Department of Microbiology & Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.
3. TASNIM Biotechnology of Research Center (TBRC), Faculty of Medicine, AJA University of Medical Sciences, Tehran, Iran.
4. Department of Microbiology, Faculty of Basic Sciences, Science and Research Branch, Islamic Azad University, Tehran, Iran.

Abstract

Background and Aims: Infectious bronchitis virus (IBV) belongs to the group of gamma coronaviruses along with other avian coronaviruses. The disease caused by IBV can appear similar to infectious laryngotracheitis, avian influenza, and velogenic Newcastle disease, which are high priority diseases. The clinical signs can be accompanied by mortalities in broiler chickens and reduced eggshell and albumin quality in layer hens, leading to economic loss for the poultry industry. Rapid detection of IBV is useful for implementation of control measures, research purposes, and understanding the epidemiology and evolution of IBVs. The aim of the present study was the rapid identification of IB with the molecular method, which targets the 5' untranslated region (UTR) gene of IBV that is less variable than the other genes, with homologies greater than 90% among IBV strains.

Materials and Methods: The primers designed to amplify a conserved fragment of the gene. Analytical sensitivity and specificity of the assay were determined.

Results: The results of specificity exhibited the specific amplification of the designed primers for IBV. Sensitivity was 10 pg/μl of the pTZ57R/T-5' UTR. This is the first report of RT-PCR method coupled with construction of comparative internal positive control (IPC) according to 5'UTR gene for accurate detection of IBV. 100 fg/μl of the IPC amplified in the presence of the limit of detection (10 pg/μl) of 5' UTR gene was determined as the optimal concentration of IPC plasmid for RT-PCR of clinical specimens.

Conclusion The RT-PCR assay presented provides a time saving, sensitive, and reliable method for detection of IBV.

Keywords: Avian Infectious Bronchitis Virus, Internal positive Control, Detection, RT-PCR

Introduction

Infectious bronchitis virus (IBV) is a Gammacoronavirus, a member of the Coronaviridae family (1). It is an enveloped, positive-sense, single-stranded RNA virus, and is highly contagious and prevalent in all countries with an intensive poultry industry, affecting the performance of both meat-type and egg-laying birds (2). IBV belongs to the group of gamma coronaviruses along with other avian coronaviruses. It is a positive-stranded RNA virus, with a genome of about 27 kb containing 5' and 3' untranslated regions (UTRs) with a poly (A) tail (3). A major part of the genome is composed of two overlapping open-reading frames (ORFs), 1a and 1b, which are translated into large polyproteins 1a and 1ab, respectively, through a ribosomal frame shift mechanism. The primary structural proteins spike (S), envelope (E), membrane (M), and nucleocapsid (N) are coded by regions found in the remaining part of the RNA genome (4, 5). IBV is the causative agent of avian infectious bronchitis (IB), which is characterised by respiratory and/or renal and/or reproductive disease (6). The disease caused by IBV can appear similar to infectious laryngotracheitis, avian influenza, and viscerotropic velogenic Newcastle disease, which are high priority diseases (7). The clinical signs can be accompanied by mortalities in broiler chickens and reduced eggshell and albumin quality in layer hens, leading to economic loss for the poultry industry and farmers. In broilers, IBV affects weight gain and feed efficiency, and, when complicated with bacterial infections like *E. coli* or *S. aureus*, it causes high mortality and increased condemnations (8-10). Many different IBV strains have been isolated and classified around the world that are extremely difficult to control because multiple serotypes

and variants of the virus occur that are not cross-protective (11, 12). So, rapid detection of IBV is useful for implementation of control measures, research purposes, and understanding the epidemiology and evolution of IBVs (13, 14).

Various vaccines and protocols to control this viral disease have been developed, although the severity of this disease varies from place to place and flock to flock (15). Live attenuated vaccines for the major serotypes of IBV can be used to control most outbreaks, but it is necessary to isolate and definitively identify the serotype of the virus responsible for the outbreak (16).

Several laboratory methods such as virus isolation in Embryonated chicken eggs (ECEs), the reference standard and organ cultures and serological tests are available for detecting and differentiating avian viral respiratory infections (17-19). However, these methods are time-consuming and laborious and may require more than one passage before obtaining a result (20, 21). Nevertheless, molecular techniques such as reverse transcription-polymerase chain reaction (RT-PCR) are rapid, specific, and accurate (22-25). Compared to virus isolation, these assays may lack sensitivity when used directly from clinical specimens. To overcome this drawback, viral RNA extraction is preceded by the propagation of the virus in chicken embryonated eggs for 36-48h (26, 27).

The aim of this study was the rapid identification of IBV with the molecular method. The 5' UTR gene was chosen since it is highly conserved among IBV strains and is abundant in infected cells. In this study, an RT-PCR targeting the 5' UTR gene for IBV detection was developed.

Methods

cDNA synthesis

IBV vaccine (Massachusetts type, H120, Merial) was candidate for PCR and preparation of positive control. CinnaPure One kit (sinaclon, Iran) was selected for total RNA extraction from the candidate vaccine using manufacture's instruction. Quality and quantity

* **Corresponding author:** Arash Ghalyanchi-Langeroudi, PhD. Department of Microbiology & Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.
E-mail: arashghalyanchi@gmail.com
Tel: (+98) 21-61117154

assessment of the extracted RNA was carried out by optical absorption measurement. Random hexamer primer was applied for cDNA synthesis according to the standard protocol.

Primer design for external positive control construction

IBV 5' UTR genes were chosen for Primer Design using primer CLC workbench version 3.5.1 (CLC Bio, Denmark). The forward primer sequence was 5' GGCTATACGACGTTTGTGA 3' and the reverse one was 5' GGAGATACTCCCTGTTTTAG 3'. Primer synthesis was done by Bioneer Co. These primers amplified a sequence of 129 bp. RT-PCR was performed at a volume of 25 µl including dNTPS (0.2 mM), MgCl₂ (1.5 mM) and 0.4 mM from each of the primers, one unit of the Taq DNA polymerase enzyme and 50 ng of cDNA. The negative control reaction was set as a reaction similar to the above but with deionized water instead of cDNA. Thermal conditions of the PCR consisted of primary denaturation at 94°C for 2 minutes, 34 cycles of denaturation at 94°C for 30 seconds, annealing at 45°C for 30 seconds, amplification at 72°C for 35 seconds.

Specificity determination of the RT-PCR assay

For determination of the specificity, PCR reactions were done according to above conditions in the presence of 50 ng of other virus's genomes such as Newcastle disease virus (B1 Type, LaSota Strain), Avian influenza virus (H9N2), infectious laryngotracheitis S(Vaccine), Infectious bursal disease (IBDL vaccine) as a negative controls. In addition, 50 ng of cDNA of IBV used a positive control.

Production of external positive control

For preparing external positive control, the PCR product was first purified using PCR purification kit (Bioneer, Korea). Ligation reaction between purified sequence and pTZ57R/T vector was done using T4 DNA ligase (Fermentas, Canada) at 22°C for 2 hours. The ligated construct was transferred to the competent *E. coli* JM107 cells. The recombinant cells were cultured on Luria

Bertani Agar (Merck, Germany) including 5-bromo-4chloro-3-indole beta digalactopyranosid (X-Gal) with 40 µg/ml, isopropyl beta-D-thiogalactopyranoside (IPTG) with a concentration of 38.4 µg/ml, ampicillin with concentration of 100 µg/ml and Nalidixic acid with the concentration of 30 µg/ml. The mentioned culture was incubated at 37°C for 16 hours. PCR on colony assay was done for selection of desired recombinant white colony. Plasmid extraction was made via AccuPrep Plasmid Mini Extraction Kit (Bioneer, Korea). Final confirmation of the insert-receiving plasmid (pTZ57R/T-5' UTR), the restriction map of the plasmid was appraised. For depiction of the map, 500ng of pTZ57RT-5' UTR plasmid was digested with 2 units of the enzyme XbaI (Fermentas, Canada).

Determination of sensitivity

In order to determine the sensitivity, pTZ57RT-16S plasmid were serial diluted (100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg and 100 fg). PCR assay was performed on all serial dilutions. The last dilution of the pTZ57RT-5' UTR plasmid yielded a detectable band on the agarose gel was assigned as the limit of detection (LOD).

Production of the internal positive control

The pTZ57R/T-5' UTR plasmid was used to create an internal positive control in accordance with study by Majidzadeh *et al* (2014) using CLC Workbench version 3.5.1 (CLC Bio, Denmark) (28). Briefly, based on the amplicom of diagnostic primers of IBV (anneal to the oligonucleotides No.1-18 (forward primers) and No. 110-129 (reverse primers). Consequently, an inner forward with MscI site at 5' end (5' TGGCCAGTTTGTAGGGGGTAGTGCC 3', anneals to nucleotides No. 12-30) and an inner reverse primer MscI site at 5' end (5' TGGCCACCCTGTTTTAGGCTTGAAG 3', anneals to nucleotides No. 102-120) was designed. The PCR reactions were prepared according to the standard protocol in total volumes of 25 µl with annealing temperature of 50°C. An inner forward primer was amplified a 118 bp fragment 1 together with diagnostic reverse primer of IBV. An inner reverse primer was amplified a 120 bp

Development of RT-PCR Using External and Internal Positive Controls ...

fragment 2 together with diagnostic forward primer of IBV. Fragment 1 and 2 were cloned in *E. coli* JM107 according to manufacturer's instruction (Fermentas, Canada). The colonies receiving each of the fragments 1 and 2 were chosen by colony PCR. Plasmid extraction was done separately using AccuPrep Plasmid Mini Extraction Kit (Bioneer, Korea). Finally, the confirmed plasmids were separately linearized with *MscI* enzyme based on standard protocol (Fermentas, Canada). Concerning the complementary sites which presences in two fragments; a ligation reaction was performed between the two linearized fragments by T4DNA ligase (Fermentas, Canada). The ligation product was cloned in the *E. coli* JM107. Screening of the desired recombinant colony (Containing fused fragment 1 and 2) was carried out with colony PCR using diagnostic forward and reverse primers of IBV. Final confirmation of IPC plasmid was applied by Cycle sequencing method using universal primers. The sequencing results were checked by CLC Workbench version 3.5.1 (CLC Bio, Denmark).

The best IPC concentration

To determine the best concentration of the IPC plasmid to apply for detection of clinical specimens, the following research was utilized. Non-infected tracheal or cloacal swabs samples were spiked with a 10-fold serial dilutions ranging 10 ng/ μ l to 10 fg/ μ l of IPC plasmid. Total RNA was extracted on specimens by a QIAamp® DNA Mini Kit (Qiagen, USA). Diagnostic PCR assay for molecular identification of IBV was done with diagnostic IBV primers in the presence of extracted IPC plasmid and 10 pg/ μ l (equal to the limit of detection of the method) of pTZ57R/T-5 UTR plasmid.

Results

Analytical specificity of the RT-PCR assay

RT-PCR assay with diagnostic primers of IBV 5' UTR showed 129 bp band. Analysis of the agarose gel related to electrophoresis of the PCR on negative control viruses' genomes using IBV 5' UTR diagnostic primers exhibited

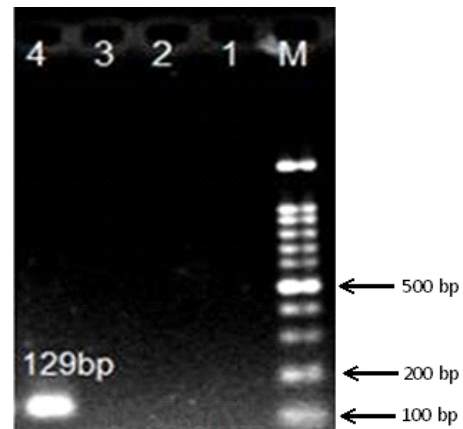


Fig. 1. The results of specificity determination of the assay. M: Marker 100 bp; 1: Negative control; 2: No amplification of Newcastle disease virus (B1 Type, LaSota Strain); 3: No amplification of Avian influenza virus (H9N2); 4: 129 bp band of IBV 5' UTR.



Fig. 2. The results of the PCR on serial dilution of external positive control. These data showed LOD of the assay equal to 10 pg/ μ l.

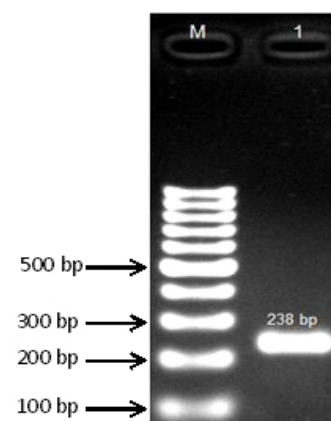


Fig. 3. The specific amplification of recombinant IPC plasmid showed 238 bp band.

no amplification. These results emphasized the specificity of the assay for IBV (Figure 1).

Analytical sensitivity of the RT-PCR assay
PCR amplification of the recombinant plasmid containing IBV 5' UTR cDNA of IBV and

Table 1: The consequences of amplification of IPC plasmid in the presence of IBV target gene.

The results exhibited the best concentration of IPC plasmid for used as an internal positive control equal to 100 fg/ μ l

Concentration of target gene	Concentration of IPC	Amplification of target gene	Amplification of IPC gene
10 pg/ μ l	10 ng/ μ l	-	+
10 pg/ μ l	1 ng/ μ l	-	+
10 pg/ μ l	100 pg/ μ l	-	+
10 pg/ μ l	10 pg/ μ l	-	+
10 pg/ μ l	1 pg/ μ l	-	+
10 pg/ μ l	100 fg/ μ l	+	+
10 pg/ μ l	10 fg/ μ l	+	-

digestion process confirmed the existence of desired gene in pTZ57R/T vector. Sensitivity determination confirmed 10 pg/ μ l concentration was the last amplifiable dilution of the pTZ57R/T-5' UTR. Hence the LOD was determined 10 pg/ μ l (Figure 2).

Internal positive control construction

Expected sizes 120 bp and 118 bp after PCR amplification of recombinant plasmid containing fragment 1 and 2 approved the existence of the mentioned products in pTZ57R/T plasmid (data not shown). Enzymatic digestion of the both fragments with MscI enzyme showed linear form of the both plasmid with predicted sizes of 3006 bp and also 3004 bp (data not shown). The colony receiving plasmid containing the complete sequence of IPC was identified by PCR using diagnostic primers of IBV. Amplification of IPC piece showed a band of 238 bp (Equal to the total of the first and second fragments sizes) (Figure 3). The resulted piece was longer than the target gene while both end having similar attachment sites to the target gene. Sequencing of recombinant plasmid containing

internal positive control piece and RFLP analysis demonstrated the presence of MscI enzyme in the IPC sequence, as expected. The minimum concentration of the IPC piece, which didn't obstruct in the RT-PCR amplification of the target gene, was introduced as the best concentration. Based on table 1, 100 fg/ μ l of the IPC amplified in the presence of the limit of detection (10 pg) of 5' UTR gene was determined as the best concentration of IPC plasmid for RT-PCR of clinical specimens.

Discussion

Viral respiratory diseases are common causes of economic losses in poultry industry (29). These diseases cause reduction of growth rate and production, high rate of death, prevention and treatment costs (30). Quick detection and differentiation of causative viruses can play an important role in controlling these viruses (31). IBV and NDV are the viruses that frequently affect the respiratory tract of chickens (32). There are several clinically similar viral

diseases that can occur in intensive poultry production and require laboratory differential diagnosis (33). Coronavirus came into the searchlight when the SARS pandemic occurred in 2003. The intense work spurred then has revealed an unexpected variety of CoV in various host species (34). The high complexity and genetic diversity of coronaviruses and the economic losses the viruses generate in breeding animals justify efforts to develop broad-spectrum detection techniques. In addition, such assays would be relevant to identify animal reservoirs of new pathogenic coronaviruses (35). Because of high sensitivity and specificity molecular method that conventional methods offer since its introduction researchers use it extensively as an indispensable diagnostic method to detect viruses (13, 36).

In this study, we developed an RT-PCR targeting a conserved region of the coronavirus 5'UTR gene developed. Targeting the 5'UTR gene has been postulated to be a better choice to improve the sensitivity of the RT-PCR as this gene is supposed to have the most abundant copy number during viral replication (37, 38). The RT-PCR assay described in this study targets the 5'UTR gene of IBV, which is less variable than other genes, with homologies greater than 90% among IBV strains (39). The primers designed to amplify a conserved fragment of the gene. It is important to rapidly differentiate IBV from disease agents like highly pathogenic avian influenza virus and exotic Newcastle disease virus, which can be extremely similar in the early stages of their pathogenesis (40). Accordingly, the specificity of our test was demonstrated by the absence of positive reaction with other RNA viruses Newcastle disease virus (B1 Type, LaSota Strain), Avian influenza virus (H9N2), infectious laryngotracheitis (Vaccine), Infectious bursal disease (IBDL vaccine) and confirmed negative for coronavirus infection. Based on serially diluted cRNA standards, the RT-PCR detected down to 10 pg/ μ l of cRNA from IBV.

Adzhar *et al.* (1996) developed universal oligonucleotides for the detection of IBV by the PCR. RT-PCR analysis using these

oligonucleotide pairs showed that a number of field isolate preparations also contained a small amount of Massachusetts (Mass) serotype virus, probably of vaccine origin and indicative of low-level persistent infection (41).

Callison *et al.* (2006) developed a real-time Taqman RT-PCR assay for the detection of IBV from infected chickens. The assay amplifies a 143-bp product in the 5' UTR of the IBV genome and has a limit of detection and quantification of 100 template copies per reaction. All 15 strains of IBV tested as well as two Turkey coronavirus strains were amplified, whereas none of the other pathogens examined, tested positive (40). Escutenaire *et al.* (2007) designed RT-PCR assay based on SYBR Green chemistry and degenerate primers were developed for the generic detection of coronaviruses. The primers, designed in the open reading frame 1b, enabled the detection of 32 animal coronaviruses including strains of canine coronavirus, feline coronavirus, transmissible gastroenteritis virus (TGEV), bovine coronavirus (BCoV), murine hepatitis virus (MHV) and IBV (42).

Shirvan & Mardani (2014) reported that the duplex-RT-PCR is a quick and sensitive procedure for simultaneously detecting IBV and NDV in birds with respiratory infections. They designed their RT-PCR reaction based on 3'UTR of IBV and M gene of NDV (43). In addition, high-resolution melt curve analysis (HRM) based on 3' UTR region can rapidly assign the majority of IBV present in field submissions to known subgroups. Importantly, HRM curve analysis also identified variant genotypes that require further investigation (44). PCR primers were designed to conserved regions within the nucleocapsid (N) gene of TCV and to the polymerase gene of TAsV-2. A multiplex RT-PCR assay was developed for the simultaneous detection of two enteric viruses of poultry: turkey enteric coronavirus (TCV) and turkey astrovirus (TAsV). PCR primers were designed to conserved regions within the N gene of TCV and to the polymerase gene of TAsV-2. The detection limit was determined to be 10 pg of RNA used as starting template (45). Meir *et al.* (2010) designed a real-time TaqMan RT-PCR assay

for the detection of IBV in chickens and compare it with RT-PCR and virus isolation. In this study, the development of a real-time TaqMan RT-PCR targeting the highly conserved N gene of IBV and including an internal PCR control is described. Real-time RT-PCR and virus isolation were 17–75% more sensitive than RT-PCR targeting the S1 gene for testing tracheal swabs directly from experimentally infected chicks. When tracheal and cloacal swabs from clinical specimens were tested directly, 50% more samples were positive by real-time RT-PCR than by the S1 gene RT-PCR (39). Acevedo *et al.* (2013) evaluated a duplex SYBR Green I-based real-time RT-PCR assay for the simultaneous detection and differentiation of Mass. and non-Mass. serotypes of IBV. The detection limit of the assay was 8.2 gene copies/mL based on in vitro transcribed RNA and 0.1 EID₅₀/mL. The assay was able to detect all the IBV strains assessed and discriminated well among the IBV Mass. and the IBV non-Mass. serotypes strains (46). To our knowledge, this is the first report of an RT-PCR method couple with constrictor of comparative IPC according to 5'UTR gene for accurate detection of IBV. The addition of an internal positive control for the detection of PCR-inhibitors allows the distinction between true and false negatives. Various PCR inhibitors such as EDTA, heparin, hemoglobin, ethanol and SDS may not remove during DNA extraction procedure and lead to reaction inhibition. Therefore, a strategy to provide the necessary information for accurate interpretation of PCR assay results is required (47, 48). To monitor the false negative results of PCR, different standards have been developed (49). According to the guidelines of international standard organization (ISO), the presence of internal positive control (IPC) in the reaction is mandatory (28, 50). IPCs are classified as competitive and noncompetitive. Noncompetitive IPCs include a group of internal controls with primer annealing sites different from target gene (28). It is recommended to use competitive IPC instead of a noncompetitive IPC. Competitive IPCs have similar primer annealing sites for the target gene and are amplified by the same

primer pairs and conditions along with the target gene. The method proved to be suitable for diagnostic purposes, showing high sensitivity and specificity when applied to biological samples from different animal species (51).

Conclusion

In conclusion, the RT-PCR assay presented in this paper provides a time-saving, sensitive, and reliable method for detection of IBV directly from tracheal or cloacal swabs, as well as in allantoic fluid from infected embryonated eggs.

Acknowledgement

This study financially supported by Vice-presidency for Research and Technology of Shahid Beheshti University, Tehran, IRAN. In addition, Iranian veterinary organization under grant (No. 22/39007) financially supported part of the project. The authors gratefully acknowledge Mr. Behrooz Asadi and Dr. Hamideh Najafi for their generous and extensive technical supports.

References

1. Liu S, Chen J, Chen J, Kong X, Shao Y, Han Z, et al. Isolation of avian infectious bronchitis coronavirus from domestic peafowl (*Pavo cristatus*) and teal (*Anas*). *Journal of General Virology*. 2005;86(3):719-25.
2. Lai MM, Cavanagh D. The molecular biology of coronaviruses. *Advances in virus research*. 1997;48:1-100.
3. Bournsnel ME, Brown TD, Foulds IJ, Green PF, Tomley FM, Binns MM. Completion of the sequence of the genome of the coronavirus avian infectious bronchitis virus. *The Journal of general virology*. 1987;68 (Pt 1):57-77.
4. Mo M, Huang B, Wei P, Wei T, Chen Q, Wang X, et al. Complete genome sequences of two Chinese virulent avian coronavirus infectious bronchitis virus variants. *Journal of virology*. 2012;86(19):10903-4.
5. Maclachlan NJ, Dubovi EJ. *Fenner's veterinary virology*: Academic press; 2010.

6. Hewson K, Noormohammadi AH, Devlin JM, Mardani K, Ignjatovic J. Rapid detection and non-subjective characterisation of infectious bronchitis virus isolates using high-resolution melt curve analysis and a mathematical model. *Archives of virology*. 2009;154(4):649-60.
7. Jackwood M, Hilt D, Callison S. Detection of infectious bronchitis virus by real-time reverse transcriptase-polymerase chain reaction and identification of a quasispecies in the Beaudette strain. *Avian diseases*. 2003;47(3):718-24.
8. Cavanagh D, Naqi S. Infectious bronchitis. *Diseases of poultry*. 2003;11:101-19.
9. Almeida D, Tortelly R, Nascimento E, Chagas M, Khan M, Pereira V. Avian infectious bronchitis and deep pectoral myopathy—A case control study. *Poultry science*. 2012;91(12):3052-6.
10. Ghareeb K, Awad W, Böhm J. Ameliorative effect of a microbial feed additive on infectious bronchitis virus antibody titer and stress index in broiler chicks fed deoxynivalenol. *Poultry science*. 2012;91(4):800-7.
11. Etteradossi N, Saif Y. Infectious bursal disease. *Diseases of poultry*. 2008;12.
12. Jackwood MW. Review of infectious bronchitis virus around the world. *Avian diseases*. 2012;56(4):634-41.
13. De Wit J. Detection of infectious bronchitis virus. *Avian pathology*. 2000;29(2):71-93.
14. Hewson K, Robertson T, Steer P, Devlin J, Noormohammadi A, Ignjatovic J. Assessment of the potential relationship between egg quality and infectious bronchitis virus infection in Australian layer flocks. *Australian veterinary journal*. 2014;92(4):132-8.
15. Callison SA. Molecular tools for the study, diagnosis, and control of infectious bronchitis virus: uga; 2003.
16. Weiss SR, Navas-Martin S. Coronavirus pathogenesis and the emerging pathogen severe acute respiratory syndrome coronavirus. *Microbiology and molecular biology reviews*. 2005;69(4):635-64.
17. Cook JK, Darbyshire J, Peters R. The use of chicken tracheal organ cultures for the isolation and assay of avian infectious bronchitis virus. *Archives of virology*. 1976;50(1-2):109-18.
18. Shan S, Ko L-S, Collins RA, Wu Z, Chen J, Chan K-Y, et al. Comparison of nucleic acid-based detection of avian influenza H5N1 with virus isolation. *Biochemical and biophysical research communications*. 2003;302(2):377-83.
19. Peng F, Wang Z, Zhang S, Wu R, Hu S, Li Z, et al. Development of an immunochromatographic strip for rapid detection of H9 subtype avian influenza viruses. *Clinical and Vaccine Immunology*. 2008;15(3):569-74.
20. Gelb Jr J, Jackwood M. Infectious bronchitis. A laboratory manual for the isolation and identification of avian pathogens, 4th ed DE Swayne, JR Glisson, MW Jackwood, JE Pearson, and WM Reed, eds American Association of Avian Pathologists, Kennett Square, PA. 1998:169-74.
21. Gelb J, Jackwood M, Swayne D, Glisson J, Pearson J, Reed W. A laboratory manual for the isolation and identification of avian pathogens. A laboratory manual for the isolation and identification of avian pathogens. 1998.
22. Cavanagh D, Brian DA, Brinton M, Enjuanes L, Holmes K, Horzinek M, et al. The Coronaviridae now comprises two genera, coronavirus and torovirus: report of the Coronaviridae Study Group. *Coronaviruses: Springer*; 1993. p. 255-7.
23. Gelb Jr J, Weisman Y, Ladman B, Meir R. S1 gene characteristics and efficacy of vaccination against infectious bronchitis virus field isolates from the United States and Israel (1996 to 2000). *Avian pathology*. 2005;34(3):194-203.
24. Jackwood MW, Yousef NMH, Hilt DA. Further development and use of a molecular serotype identification test for infectious bronchitis virus. *Avian diseases*. 1997:105-10.
25. Lee C-W, Jackwood MW. Origin and evolution of Georgia 98 (GA98), a new serotype of avian infectious bronchitis virus. *Virus Research*. 2001;80(1):33-9.
26. Kwon HM, Jackwood MW, Gelb Jr J. Differentiation of infectious bronchitis virus serotypes using polymerase chain reaction and restriction fragment length polymorphism analysis. *Avian diseases*. 1993:194-202.
27. Meir R, Rosenblut E, Perl S, Kass N, Ayali G, Hemsani E, et al. Identification of a novel nephropathogenic infectious bronchitis virus in Israel. *Avian diseases*. 2004;48(3):635-41.
28. Majidzadeh K, Mohseni A, Soleimani M. Construction and evaluation of a novel internal positive control (IPC) for detection of *Coxiella burnetii* by PCR. *Jundishapur Journal of Microbiology*. 2014;7(1).
29. Dekich M. Broiler industry strategies for control of respiratory and enteric diseases. *Poultry science*. 1998;77(8):1176-80.
30. Cavanagh D. Coronavirus avian infectious bronchitis virus. *Veterinary research*. 2007;38(2):281-97.
31. Purvis LAB. MOLECULAR DETECTION AND CHARACTERIZATION OF INFECTIOUS BURSAL DISEASE AND CHICKEN ANEMIA

VIRUS FROM COMMERCIAL BROILERS. 2007.

32. Roussan D, Haddad R, Khawaldeh G. Molecular survey of avian respiratory pathogens in commercial broiler chicken flocks with respiratory diseases in Jordan. *Poultry science*. 2008;87(3):444-8.
33. Dhama K, Singh SD, Barathidasan R, Desingu P, Chakraborty S, Tiwari R, et al. Emergence of avian infectious bronchitis virus and its variants need better diagnosis, prevention and control strategies: A global perspective. *Pakistan Journal of Biological Sciences*. 2014;17(6):751.
34. Mackenzie J, Drury P, Ellis A, Grein T, Leitmeyer K, Mardel S, et al., editors. The WHO response to SARS, and preparations for the future. *Learning from SARS: Preparing for the Next Disease Outbreak: Workshop Summary*; 2004.
35. Siddell SG, Ziebuhr J, Snijder EJ. Coronaviruses, toroviruses, and arteriviruses. *Topley and Wilson's microbiology and microbial infections*. 2005.
36. Dong J, Olano JP, McBride JW, Walker DH. Emerging pathogens: challenges and successes of molecular diagnostics. *The Journal of Molecular Diagnostics*. 2008;10(3):185-97.
37. Brian DA, Baric R. Coronavirus genome structure and replication. *Coronavirus replication and reverse genetics*: Springer; 2005. p. 1-30.
38. van der Hoek L, Pyrc K, Jebbink MF, Vermeulen-Oost W, Berkhout RJ, Wolthers KC, et al. Identification of a new human coronavirus. *Nature medicine*. 2004;10(4):368-73.
39. Meir R, Maharat O, Farnushi Y, Simanov L. Development of a real-time TaqMan® RT-PCR assay for the detection of infectious bronchitis virus in chickens, and comparison of RT-PCR and virus isolation. *Journal of virological methods*. 2010;163(2):190-4.
40. Callison SA, Hilt DA, Boynton TO, Sample BF, Robison R, Swayne DE, et al. Development and evaluation of a real-time Taqman RT-PCR assay for the detection of infectious bronchitis virus from infected chickens. *Journal of virological methods*. 2006;138(1):60-5.
41. Adzhar A, Shaw K, Britton P, Cavanagh D. Universal oligonucleotides for the detection of infectious bronchitis virus by the polymerase chain reaction. *Avian Pathology*. 1996;25(4):817-36.
42. Escutenaire S, Mohamed N, Isaksson M, Thoren P, Klingeborn B, Belák S, et al. SYBR Green real-time reverse transcription-polymerase chain reaction assay for the generic detection of coronaviruses. *Archives of virology*. 2007;152(1):41-58.
43. Shirvan AS, Mardani K, editors. Molecular detection of infectious bronchitis and Newcastle disease viruses in broiler chickens with respiratory signs using Duplex RT-PCR. *Veterinary Research Forum*; 2014: Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.
44. Hewson K, Browning G, Devlin J, Ignjatovic J, Noormohammadi A. Application of high-resolution melt curve analysis for classification of infectious bronchitis viruses in field specimens. *Australian veterinary journal*. 2010;88(10):408-13.
45. Sellers HS, Koci MD, Linnemann E, Kelley LA, Schultz-Cherry S. Development of a multiplex reverse transcription-polymerase chain reaction diagnostic test specific for turkey astrovirus and coronavirus. *Avian diseases*. 2004;48(3):531-8.
46. Acevedo AM, Perera CL, Vega A, Ríos L, Coronado L, Relova D, et al. A duplex SYBR Green I-based real-time RT-PCR assay for the simultaneous detection and differentiation of Massachusetts and non-Massachusetts serotypes of infectious bronchitis virus. *Molecular and cellular probes*. 2013;27(5):184-92.
47. Wilson IG. Inhibition and facilitation of nucleic acid amplification. *Applied and environmental microbiology*. 1997;63(10):3741.
48. BICKLEY J, HOPKINS D. Inhibitors and Enhancers of PCR. *Analytical Molecular Biology: quality and validation*. 1999;8:81.
49. Klein D. Quantification using real-time PCR technology: applications and limitations. *Trends in molecular medicine*. 2002;8(6):257-60.
50. Amoroso MG, Salzano C, Cioffi B, Napoletano M, Garofalo F, Guarino A, et al. Validation of a Real-time PCR assay for fast and sensitive quantification of *Brucella* spp. in water buffalo milk. *Food control*. 2011;22(8):1466-70.
51. Das A, Spackman E, Senne D, Pedersen J, Suarez DL. Development of an internal positive control for rapid diagnosis of avian influenza virus infections by real-time reverse transcription-PCR with lyophilized reagents. *Journal of clinical microbiology*. 2006;44(9):3065-73.