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**Original Article**

# Determination of RNA genome in the low titer zoonotic RNA virus samples

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**Abstract**
There are numerous RNA virus infections in mammals as emerging zoonosis originating from wildlife. The total viral diversity is unknown. The total number of mammalian viruses is estimated to be more than 320000. Many of these viruses have genomic RNA and are important as zoonotic agents. In many RNA virus infections, the virus load in the serum can be used as a marker for severity. For diagnostic purposes dealing with the low virus titer in serum and biotechnological applications, it is mandatory and highly important, to have a native, innovative, and sensitive RNA isolation kit. For this aim, we used an RNA isolation kit with RNA carrier, produced by Research Institute Molecular Biological System Transfer (MBST, Tehran, Iran) and the Avian infectious bronchitis virus vaccine of Nobilis, 2500 Doses, $10^3$ID50 (Intervet, Netherlands) as virus RNA probe. The RNA was extracted from diluted solutions (up to $10^{-9}$). Subsequently, cDNA was synthesized. The cDNA was then amplified using a specific primer pair derived from the RNA genome of the Avian infectious bronchitis virus. Our results showed that it was possible to detect RNA viruses in the prepared samples with virus titers of up to $10^{-9}$ or 0.00001 × ID50. These results were confirmed by the Iranian National Reference Laboratory, Diagnosis and Applied Studies Center, and Veterinary Organization using their evaluation matrix Avian influenza virus sample. In conclusion, this kit is suitable for samples with low RNA virus titers.
***Keywords***: RNA virus, RT-PCR, Carrier RNA, Avian infectious bronchitis virus, Avian influenza virus

## Introduction

Zoonotic diseases are of great importance in the public and animal health management. Jones et al. (2008) reported that more than 60% of human infectious diseases belonged to zoonotic diseases between 1940 and 2004, which led to high morbidity, mortality, and economic costs. They also reported that about 71.8% of the mentioned diseases are from wildlife, and 22.8% are arthropod vector-borne infections. It is assumed that ticks

play the most important role in the transmission of zoonotic diseases within arthropods (Jongejan and Uilenberg, 2004; Colwell et al., 2011; Pfäffle et al., 2013). Qin et al. (2023) reported a total of 28 RNA viruses belonging to 12 viral families in Haemaphysalis concinna, Dermacentor silvarum, and Ixodes persulcatus in north China, which clearly shows the important role of RNA viruses in the transmission of diseases by ticks. Anthony et al. (2013) estimated that the total number of mammalian viruses can exceed 320000. Many of these viruses have RNA as genetic material. Dilcher et al. (2015) found Rhabdovirus, a negative-sense, single-stranded RNA virus with a genome of about 10.8–16.1 kb, in Hyalomma anatolicum ticks collected in Zahedan, Iran. Maghsood et al. (2020) showed the presence of Crimean-Congo Haemorrhagic Fever (CCHF) virus in Hyalomma marginatum in Khorasan Province in Iran and Telmadarraiy et al. (2015) detected CCHF virus in Rhipicephalus sanguineus, Hyalomma marginatum, H. anatolicum, H. asiaticum and H. dromedarii. Cevik et al. (2007) reported that viral loads of CCHF virus in the serum samples could be very important for determining the severity of infection. They reported that in 8 out of 9 patients with fatal outcomes, the viral loads were ≥109 copies/mL, and in 25 out of 26 patients with nonfatal outcomes, the viral loads were under 109 copies/mL. Patients with nonfatal outcomes have a mean peak virus load ≤4.1 × 106 copies/mL, whereas patients with fatal outcomes have a mean peak virus load of 7.1 × 109. Duh et al. (2007) also reported that the serum virus load could be considered in patients infected with the CCHF virus as a marker for moderate, severe, and fatal outcomes. Since many RNA viruses belong to the causative agents of emerging zoonotic infections, it is very important to have a native, innovative, and sensitive RNA isolation kit for diagnostic purposes for infected humans, animals, or arthropods as transmitters. In the present study, we have tested the RNA isolation Kit of MBST (Iran) to detect RNA virus and its sensitivity using the Avian infectious bronchitis virus vaccine of Nobilis, 2500 Doses, 103ID50 (Intervet, Netherlands) as an RNA virus probe.

## Materials and methods

*RNA extraction and cDNA synthesis*

The RNA was extracted from the Avian infectious bronchitis virus vaccine of Nobilis, 2500 Doses, 103ID50 (Inervet, Netherlands). To achieve our goal, we began by dissolving the vaccine contents in the virus vial with 2.5 mL of DEPC double distilled water. We then diluted the solution to a total volume of 1 mL, making it $10^{-9}$ in concentration. After that 100 µL of each virus diluted solution ($10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$, $10^{-7}$, $10^{-8}$, and $10^{-9}$) was used for RNA extraction. The RNA extraction was performed according to the manufacturer's protocol. Briefly, 400 µL of LB-PS was added to 100 µL diluted solution and immediately mixed thoroughly with vortexing for 10 seconds. Subsequently, 6 µL of ß-Mercaptoethanol was added to the samples and mixed thoroughly by vortexing for 10 seconds, and the mixtures were incubated for 5 min. at 70°C. After that, the vials were vortexed for 10 seconds at RT. Followed by the addition of 30 µL RNA Carrier solution to the mixtures. The mixture was mixed thoroughly by vortexing. After that, 480 µL Ethanol (96 – 100%) was added to the samples and mixed thoroughly by vortexing. 900 µL of the mixture was applied into the column and centrifuged at 8000 × g for 1 minute. The spin columns were then washed once with 500 µL WB-SPI and twice with 500 µL WB-PSII at 8000 × g for 1 minute.

Finally, the adsorbed RNA on the column was eluted with 40 µL DEPC-H2O preheated to 70°C. The cDNA was synthesized with eight µL RNA using a cDNA synthesis kit and hexa nucleotides purchased by CinaClone (Iran) in a total of 20 µL. Four µL of cDNA was then used for RT-PCR. The RT-PCR was done with 0.5 µL each primer (20 µM, MWG, Germany) primers (5` GATTATGATTATTATCGTTATAA 3` and 5` TTAGGATAATCCCAACCCAT 3`) derived from Avian infectious bronchitis virus genome

registered under Accession number NC_001451 in GenBank and master mix (CinaClone, Iran) in total volume of 25 µL in automated thermocycler (Bio-Rad, Germany) with following program: 5 min incubation at 95°C to denature cDNA, 38 cycles of 45 s at 94°C (denaturing step), 45 s at 48°C (annealing step) and 45 s at 72°C (extension step). Finally, PCR was completed with an additional extension step for 10 min. The visualization of the RT-PCR product was performed by agarose gel electrophoresis using ethidium bromide and a UV transilluminator. The PCR product should have a length of 512 bp.

As a control, three Avian infectious bronchitis-positive field samples, a generous gift from Prof. Dr. Mahdi Vasfi Marandi (deceased), Department of Avian Health and Diseases, Faculty of Veterinary Medicine, University of Tehran were also used.

For evaluation of the RNA extraction kit, the kit was sent to the Iranian National Reference Laboratory, Diagnosis & Applied Studies Center, Iran Veterinary Organization, where the kit was evaluated using their evaluation matrix Avian influenza RNA virus. For the cDNA synthesis and RT-PCR, SuperScript®III Platinum® One-Step q RT–PCR System was used.

Qty: 500 rxn, Lot: 2422210, REF: 11732-088.

*Positive control construct*

To control the primers, cDNA synthesis, and PCR, a DNA fragment was cloned in plasmid pEX128A by MWG (Germany), flanking the mentioned primers. The PCR with this recombinant plasmid resulted in a PCR product of 545 bp in length.

**Results**

The RNA was extracted from control samples and Nobilis, a vaccine for Avian infectious bronchitis virus (Inervet, Netherlands), followed by cDNA synthesis. The cDNAs prepared with field samples and the recombinant plasmid DNA were amplified using the primers derived from the Avian infectious bronchitis virus genome. It is to denote that this study cannot be considered an epidemiologic study and was made do with the Avian infectious bronchitis virus vaccine and evaluation matrix Avian influenza RNA virus samples we had available. Despite these, 3 viral field samples were also used. Figure 1 shows the expected RT-PCR product of 512 bp in length by three samples prepared from the field and an RT-PCR product with 545 bp in length for recombinant pEX128A.

The results of RT-PCR with the cDNAs generated with the isolated RNA from different diluted Avian infectious bronchitis virus vaccines showed that the RT-PCR product of 512 bp in length could be observed in dilution samples up to 10-6 without RNA carrier. In order to enhance the RNA isolation capacity, we used RNA-carrier. In the case of RNA isolation with the carrier-RNA, the detection of RT-PCR products increased to the ten power of -7, which is approximately 0.0001 × ID50 (Figure 2). The mentioned results are given regardless of the dilutions during the test; taking into account the dilutions performed during the test, the sensitivity of the RNA extraction was shown to be approximately 1 × 10-8 without RNA carrier, namely 0.00001 × ID50 and 1 × $10^{-9}$ namely 0.000004 × ID50 with RNA carrier.

The mentioned RNA isolation kit was also evaluated by the Iranian National Reference Laboratory, Diagnosis & Applied Studies Center, Iran Veterinary Organization, with their evaluation matrix Avian influenza RNA virus. They used the one-step cDNA-RT-PCR method and showed that the RNA isolation kit could isolate enough RNA from up to 10-8 dilutions of the evaluation matrix sample. This result was identical to the results achieved with their reference RNA isolation kit.

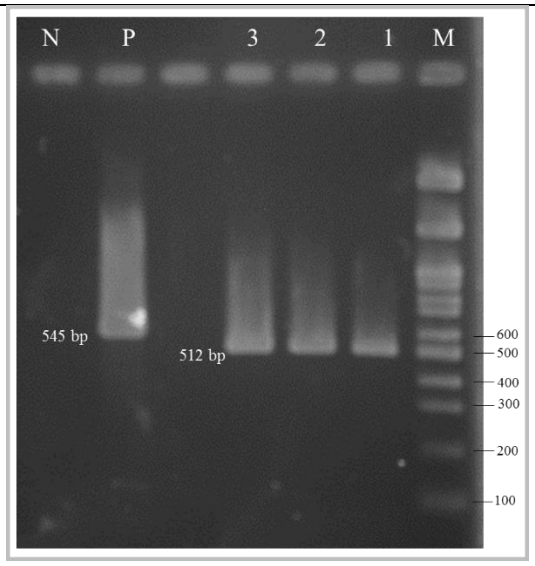


**Fig. 1.** RNA was isolated from three Avian infectious bronchitis virus samples, and the cDNA was prepared. The cDNAs (Lanes 1, 2, and 3), and the recombinant plasmid pEX128A were amplified using a primer pair derived from the genome registered under Accession number NC_001451in GenBank. N is negative control, P is plasmid control and M is 100 bp DNA marker.

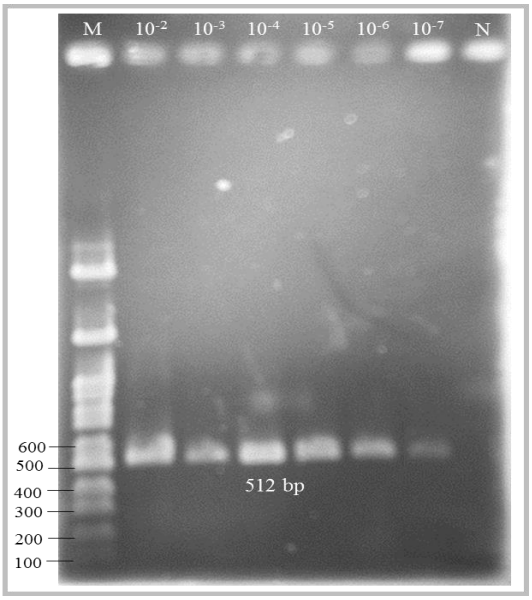


**Fig.2.** RNA was isolated from dilution series (10-1, 10-2, 10-3, 10-4, 10-5, 10-6, 10-7) Avian infectious bronchitis virus vaccine of Nobilis, 2500 Doses, 103 ID50 and the cDNA was prepared. The cDNAs were amplified using a primer pair derived from the genome registered under Accession number NC_001451in GenBank. N is the negative control, and M is the 100 bp DNA marker.

## Discussion

Viruses play an important role in the management of public and animal health, and every year, this is accompanied by a lot of financial losses in the livestock industry. Therefore, the gold standard disease management methods could be mostly restricted to early protection, diagnosis, and therapy. The outbreak of the severe acute respiratory syndrome Coronavirus-2, commonly known as COVID-19, is a notable example. It was first reported on December 31, 2019, by Wang et al. (2020) in China and quickly spread around the world. On January 30, 2020, the World Health Organization (WHO) declared it a significant global health emergency. The COVID-19 infection has caused 769,369,823 confirmed cases and 6,954,336 deaths worldwide, as reported on the WHO coronavirus (COVID-19) Dashboard on August 9, 2023. An early and specific diagnosis using molecular techniques like RT-PCR or Real-Time RT-PCR can significantly prevent the spread of the disease. Another example is the Crimean hemorrhagic fever virus, first described in 1944 in Crimea and later in 1956 in Congo (WHO, 2017). This virus can be transmitted by different Ixodid ticks and the ticks can act as reservoirs for this virus (Messina et al., 2015; Maghsood et al., 2020). The CCHF can infect wild and domestic animals including sheep, goats, cattle, and camels asymptomatically (Spengler et al., 2016a) enabling the infected animal's reservoirs but in humans, cause severe, often fatal infection. There are some reports dealing with the experimental infection of wild animals and domestic animals (Gonzalez et al., 1998; Spengler et al., 2016b), but unfortunately, we do not know exactly about the pathophysiology of this virus in domestic animals. Hongzhao et al. (2023) infected sheep with the highly pathogenic clinical isolate, and they found that the sheep did not show remarkable clinical signs. The animals showed fiver, and the virus could be detected within day two and day six after infection in the blood. The peak of virus detection was around day 5. It is interesting to note that the genome of the virus was found to be present in various tissues of the infected sheep for a

prolonged period of time. This is a matter that requires attention, particularly in our country where different types of ticks transmit the virus.

It has been reported that the virus load in the patients can be considered as a marker for severity (Cevik et al., 2007); a serum viral titer of 102-104 copies/mL could be considered moderate, whereas 104-107 copies/mL as severe and 108-1010 copies/mL as lethal. The European Network for the Diagnosis of Imported Viral Diseases (ENIVD) has released the results of an external quality assessment for Crimean-Congo Hemorrhagic Fever (CCHF) molecular diagnosis. The assessment involved 44 laboratories from 29 different countries(Escadafal et al., 2012). They emphasize that information about the serum load of the virus is crucial in pathogenesis studies and monitoring the progression of the disease. The assessment was conducted using multiple samples and serial 10-fold dilutions. The results obtained from different countries were not the same, and in many cases, there were significant differences. Only 35% of the exams were evaluated optimally, and 19% were evaluated as acceptable because, in one positive case, they could not detect the virus by RT-PCR. Interestingly, 43% of results were accompanied by several false negatives and false positives. The final evaluation showed that there is room for serious correction of the diagnosis method like better design of the primers, and use of more effective technical methods like Real-Time RT-PCR, RNA isolation kits, enzymes, and others. Interestingly, it was shown that there is a clear negative correlation between the RNA detection and dilution of the samples, which could be due to an improper RNA extraction kit.

Therefore, it is important to have sensitive tools for detecting such viruses also in low titer. The current study utilized the RNA extraction kit from MBST, Iran. Since no access to the other defined RNA vaccines, like the whole CCHF vaccine was available, the Avian infectious bronchitis virus vaccine Nobilis, 2500 Doses, 103ID50 (Inervet, Netherlands) as an RNA virus sample was used. As PCR control, recombinant vector pEX128A and three infectious bronchitis field samples were used. Our results showed that the used RNA isolation kit with RNA carrier could isolate enough RNA for diagnostic purposes from up to $10^{-7}$ dilutions of vaccine content, which is approximately 0.0004 × ID50. Taking the dilution process performed during the experiment, the sensitivity will be increased by 100 power more.

Iranian National Reference Laboratory, Diagnosis, and Applied Studies Center, and Iran Veterinary Organization also evaluated the presented RNA isolation kit using their evaluation matrix for Avian influenza RNA virus with the one-step cDNA-RT-PCR method. They could show that the RNA isolation kit could isolate enough RNA from up to $10^{-8}$ dilutions for diagnostic purposes.

## Conclusion

We believe this kit is sensitive enough to diagnose viral diseases with low titers.

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## Conflict of Interests

The authors declare no conflict of interest.

## Ethical approval

Not applicable.

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