Biomacromol. J., Vol. 3, No. 2, 100-106, December 2017.

Biomacromolecular Journal
www.bmmj.org

Comparison between RT-PCR, NASBA and RT-LAMP Methods for Detection of *Coxsackievirus* B3

M. Zeinoddini*, A. Monazah and A.R. Saeedinia

Department of Bioscience and Biotechnology, Mallek Ashtar University of Technology, Tehran, Iran

(Received 3 January 2018, Accepted 7 April 2018)

ABSTRACT

Viral myocarditis is a moderate disease, but it sometimes causes progressive cardiac disorder. Many different viruses have been considered as the agent of viral myocarditis, but *Coxsackievirus* of the B group, in particular of the *Coxsackievirus* B3 (CVB3), is more than fifty percent of cases of viral myocarditis. CVB3 is a positive single-stranded RNA virus and a member of the genus *Enterovirus* and it is most commonly causing of human viral myocarditis or human acute, especially in young patients. The goal of this study is a comparison of three molecular methods included RT-PCR, NASBA and RT-LAMP for detection of CVB3. For this purpose, the primer explorer V4 software was used for designing of specific primers. Total RNA extracted from CVB3-infected HeLa cell line after 24 h and stored in -80 °C since using as the template in RT-LAMP, NASBA and RT-PCR assays. Then, for evaluated of the sensitivity of these methods, serial dilution of total RNA was performed. The result of this study showed that the sensitivity of RT-LAMP, NASBA and RT-PCR were 0.1, 10 and 10 pg, respectively. Based on the results that obtained in this study, the RT-LAMP assay was highest sensitive than RT-PCR and NASBA techniques for detection of CVB3 infection.

Keywords: CVB3, RT-PCR, RT-LAMP, NASBA, RNA extraction, Myocarditis

INTRODUCTION

Viral myocarditis is one of the major causes of sudden deaths in adolescents. Although most people recover from acute viral myocarditis, people with this condition may develop chronic coronary artery disease, chronic myocarditis, and heart failure associated with autoimmune responses [1-4]. Several viruses can cause viral myocarditis, including *Enteroviruses*, *Cytomegalovirus*, *Adenovirus*, *Parvovirus* B19, *Hepatitis* C virus [5]. *Enteroviruses* include a group of positive RNA viruses that pass through the fecal-oral route. These viruses replicate after entering the gastrointestinal tract and then go through the blood to target organs and cause disease [6]. Group B *Coxsackieviruses* include six serotypes in the genus *Enterovirus*, with a wide range of human diseases such as acute myocarditis, aseptic meningitis, encephalitis, Bornholm disease, apnea and febrile illness [7]. Among these viral, *Coxsackievirus* of the B group, in particular of the *Coxsackievirus* B3 (CVB3), are more than fifty percent of cases of viral myocarditis and are known to be the dominant cause of viral myocarditis in humans [1,8]. Until now, much nucleic acid-based detection has developed for CVB3 diagnostic. RT-PCR (Reverse transcription-polymerase chain reaction) has used as a common diagnostic method for identification of pathogenic RNA virus agents. However, RT-PCR is commonly used in comparisons and diagnostic studies [9,10] and this method is depended to thermocycler. For this, isothermal nucleic acid amplification could be used as an alternative method for simple and sensitive diagnostic studies. NASBA (Nucleic acid amplification techniques) and LAMP (Loop-mediated isothermal amplification) are two main isothermal detection methods. NASBA is highly sensitive tools for the detection and amplification of RNA targets and developed for detecting specific nucleic acids [11]. The NASBA method is mimicking retroviral RNA replication and using of two specific oligonucleotide primers (one containing a

*Corresponding author. E-mail: zeinoddini@yahoo.com

T7 promoter sequence) and three enzymes, AMV reverse transcriptase, RNase H and T7 RNA polymerase [11-15]. In this reaction accumulates RNA and cDNA copies of the original RNA target, indicating that newly synthesized cDNAs and RNAs function as templates for a continuous series of transcription and reverse transcription reactions. In NASBA assay 10^6 fold amplification occurs after 1-2 h incubation [16]. On the other hand, loop-mediated isothermal amplification (LAMP) that firstly, has developed by Notomi in 2000 amplifies DNA with rapidity, high specificity, and efficiency under isothermal conditions. The LAMP method utilizes Bst DNA polymerase with strand displacement activity and a set of 4 specially designed primers (two inner and two outer primers) that recognize a total of 6 distinct sequences on the target DNA. The cycling reaction continues with generates of 10^9 copies of the target sequence in less than one hour. Since in the LAMP method recognizes the target by 6 different sequences initially and by 4 distinct sequences afterward, this method is high target sequence specificity [17-19]. Also, RT-LAMP (reverse transcription-LAMP) method which is carried out in a single tube is a simple, cost-effectiveness, high specific, less time-consuming and rapid method [20]. The high speed of RT-LAMP assay than conventional RT-PCR method has been used successfully for rapid detection of *Enteroviruses* as pathogenic RNA viruses [20-31]. The present study is conceptualized to detect CVB3 viruses by comparison of nucleic acid-based methods (conventional RT-PCR, NASBA, and RT-LAMP).

MATERIALS AND METHODS

Viral Culture and RNA Extraction

RPMI 1640 medium supplemented with 10% FBS was used for growth and maintenance of HeLa cell cultures. Cells at 90-100% confluency (Fig. 1A) were infected with CVB3 (Nancy strain) in a medium containing 1% FCS. After 24 h and observed complete cytopathic effect (CPE) (Fig. 1B), the infected-HeLa cells were harvested at 5000 rpm for 5 min. The cell sediment was used for total RNA extraction. For this, after two cycles of freezing and thawing, total RNA was extracted using RNX reagent according to the manufacturers' instruction. The integrity of the extracted RNA was assessed by resolution on 1% agarose gel and ethidium bromide staining. RNA samples were stored at -80 °C.

RT-PCR Assay

RT-PCR assay was carried out in a total 10 µl reaction volume containing 5 µl of 10X PCR buffer, 1 µM of forward primer (F3: 5′-GGAGAGTTGCGGATACCGT-3′) and reverse primer (B3: 5′- TCAGGTGCCAAGCGGTAT-3′), 2 mM of $MgSO_4$, 1.5 mM of dNTP, 1 mM of DTT, 5 U of RNase inhibitor, 5 U of cloned AMV reverse transcriptase (Invitrogen), 2.5 U of Taq DNA polymerase, 2 µg of template extracted total RNA. The reactions were incubated at 60 °C for 30 min, followed by initial denaturation at 94 °C for 4 min, 35 cycles of 94 °C for 60 sec, 60 °C for 60 s and 72 °C for 45 s and a final extension at 72 °C for 5 min. The RT-PCR procedure was carried out using an automated thermal cycler (Techgene, Germany) and all PCR products were analyzed by 1% agarose gel electrophoresis.

NASBA Assay

The reaction mixture of NASBA was prepared in a total volume of 20 µl containing 5 µl of extracted RNA, 40 mM Tris (pH 8.5), 12 mM $MgCl_2$, 70 mM KCl, 5 mM dithiotritol (DTT), 1 mM of each deoxyribonucleoside triphosphate (dNTP), 2 mM each ribonucleoside triphosphate, 15% (v/v) dimethylsulphoxide (DMSO) and 5 pmol of each primer (P1: 5′-CACACTACCGGTTTGTTGCTTCAG-3′, P2: 5′-TCCCTCGGTCCAAAACACACTG-3′, P3: 5′ -AATTCTAATACGACTCACTATAGGGAGAGACGTCA TATTGCGGCATGGC-3′ and P4: 5′-TTGTACCGCTAGATTACTGC-3′). The reaction mixture was incubated at 65 °C for 5 min and subsequently transferred to 41 °C. After 5 min, an enzyme mixture containing 2.1 µg of BSA, 0.08 U of RNase H (0.08 U μl^{-1}), 32 U of T7 RNA polymerase (32 U μl^{-1}) and 6.4 U of AMV-RT (Avian Myeloblastosis Virus - Reverse Transcriptase, 6.4 U μl^{-1}) was added to the mixture and the resulting reactions were performed at 41 U μl^{-1} for 90 min. NASBA products were analyzed by agarose gel electrophoresis and visualized with ethidium bromide staining.

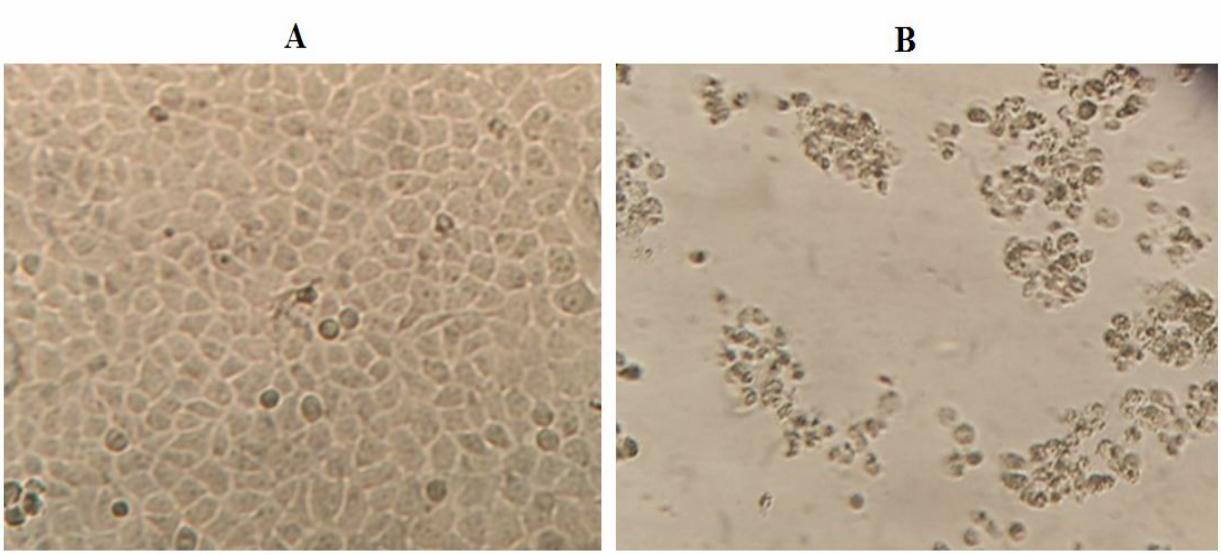

Fig. 1. CVB3 culture in HeLa cell: (A) Normal cell line and (B) Cell line showing CPE (cytopathic effect) after 24 h infected with CVB3.

RT-LAMP Assay

The CVB3 RT-LAMP assay was carried out in a total 12.5 µl-volume reactions containing1.4 mM dNTPs, 1.25 µl of 10X isothermal amplification buffer, 4 mM of $MgSO_4$, 1.25 µl of DTT (0.1 M), 0.4 µl of RNasin 0.8 M of betaine, 0.8 µM of the inner primers (FIP: 5′-CTGCTGCTGAGACGGGTCACACTTTTAGGGCCAAC CAACTCAGA-3′ and BIP: 5′-TGCAGACACGCCACGTTAAGAATTTTTGTAGGTCA GCATGCGTGTA-3′), 0.1 µM of the outer primers (F3: 5′-GGAGAGTTGCGGATACCGT-3′ and B3: 5′-TCAGGTGCCAAGCGGTAT-3′), 4 U of Bst 2.0 DNA polymerase (New England Biolabs), 5 U of cloned AMV reverse transcriptase (Invitrogen) and 10 µg of extracted total RNA. The mixture was incubated at 60 °C for 90 min and then heated at 80 °C for 10 min to stop of the reaction. To visualize the RT-LAMP products, the amplicon was analyzed by running 2 µl of the amplified product on a 2% agarose gel and ethidium bromide staining for 60 min at 60 volts in 1X TAE (Tris Acetic acid EDTA) buffer and visualized under a gel documentation system. Also, amplified RT-LAMP products were detected by adding 4 µl of 1:10 diluted 10,000x concentration of SYBR Green I to each reaction tube. The amplicon was observed by placing the reaction tube under UV irradiation source.

Selectivity and Sensitivity of RT-PCR, NASBA, RT-LAMP Assays

The specificity of RT-PCR and RT-LAMP assay was determined by CVB3 and some serotypes of genus *Enteroviruses* include of *Coxsackievirus* A16, *Echovirus 36* and *Rhinovirus*. Also, in order to evaluate the sensitivity of RT-PCR, NASBA and RT-LAMP assay for detection of CVB3, 7 concentrations of RNA from 10^6 pg μl^{-1} to10^{-5} pg μl^{-1} were prepared and used as a template for RT-PCR and RT-LAMP assay, and 7 concentrations of RNA from 10^5 pg μl^{-1} to 1 pg μl^{-1} were prepared and used as a template for NASBA assay. Then, the amplified DNA was analyzed using 2% agarose gel electrophoresis.

Also in order to evaluate the specificity of RT-PCR and RT-LAMP method was determined by CVB3 and some serotypes of genus *Enteroviruses* include of *Echovirus 36*, *Coxsackievirus* A16 and *Rhinovirus*.

RESULTS

In this study, the RT-PCR, NASBA and RT-LAMP assays were performed for the detection of CVB3 in total RNA extracted from virus-infected cell culture. The results of RT-LAMP and NASBA were compared with RT-PCR.

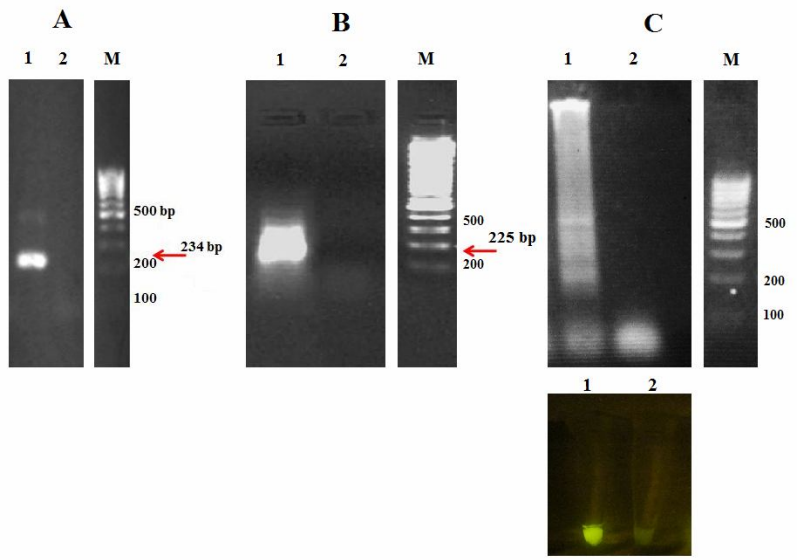

Fig. 2. Products of RT-PCR (A), NASBA (B) and RT-LAMP (C) reactions. Lane 1: positive control, lane 2: negative control and lane M: 100 bp DNA leader.

Virus Detection in Cell Culture by RT-PCR, NASBA and RT-LAMP Assays

Total RNA extracted from CVB3 infected cells was used to test the sensitivities of the RT-PCR, NASBA and RT-LAMP assays. The size of NASBA and RT-PCR products were 234 and 225 bps and RT-LAMP products were observed as ladder-like patterns on the agarose gel (Fig. 2).

Sensitivity and Selectivity of RT-PCR, NASBA and RT-LAMP Assays

In order to evaluate the sensitivity of RT-PCR, NASBA and RT-LAMP detections of CVB3, we used 7 deferent concentrations of total RNA from virus-infected cells. The data have shown that the sensitivity of RT-PCR, NASBA, and RT-LAMP were 10, 10 and 0.1 pg, respectively. Therefore, the sensitivity of RT-LAMP assay was 100 fold higher than RT-PCR and NASBA (Fig. 3). Also, the selectivity of RT-PCR and RT-LAMP with serotypes of genus *Enteroviruses* indicated that designed primers are very specific (Fig. 4).

DISCUSSION

Coxsackieviruses are major cell pathogens that cause a wide variety of human diseases from simple colds to lethal myocarditis. These viruses usually enter the body through the gastrointestinal tract and multiply in the throat, small streams and lymph nodes as the first regions. Then enter the bloodstream or the lymph system and enter the target organ [23]. Various methods such as RT-PCR [32], NASBA technique [12], and in situ hybridization [33], were using for detection of CVB3 in heart patients. Diagnostic methods that are commonly used to detect *Enterovirus* infections include cell culture and culture of these viruses, followed by the use of serum neutralization tests [34]. Although the methods are very reliable, their disadvantage is that they are the time-consuming and costly task. In addition, the supply of antiserum is limited to them, and the specific diagnosis of the type of enteroviral infection is not possible with this method [23]. Isothermal amplification methods, such as NASBA and LAMP in contrast to the conventional PCR method, which require a large cycle to amplification of the target segment, can be amplified by incubation of the reaction mixture at a single temperature at the same time [35].

In this study molecular detection methods include RT-PCR, NASBA and RT-LAMP assays are successfully applied in the detection of CVB3. The aim of this study was to compare the sensitivity of RT-PCR, NASBA and RT-

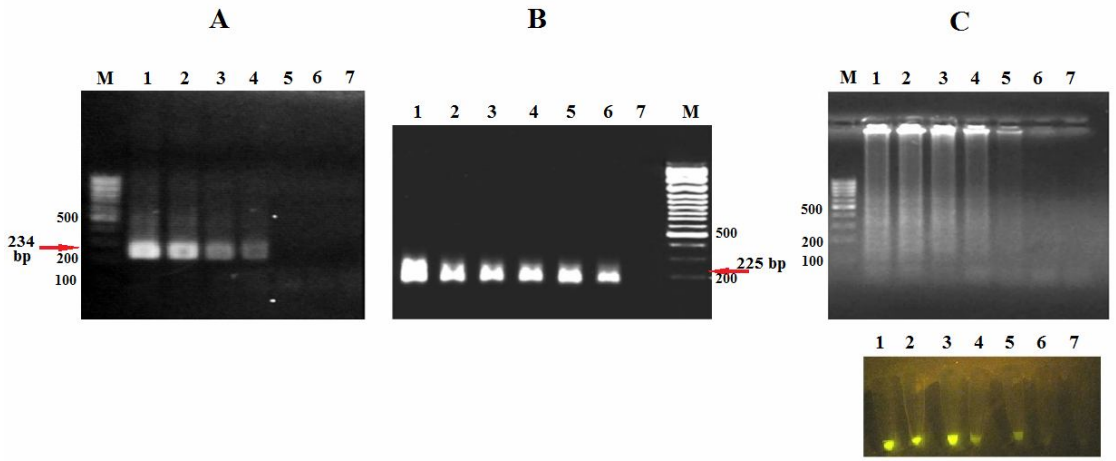

Fig. 3. Sensitivity of RT-PCR (A), NASBA (B) and RT-LAMP (C) reactions. Sensitivity of reactions was determined by serial dilutions of total RNA from virus infected cells. (A) RT-PCR; Lane 1: 10^{6} pg, lane 2: 10^{4} pg, lane 3: 10^{2} pg, lane 4: 10 pg, lane 5: 10^{-1} pg, lane 6: 10^{-3} pg and lane 7: 10^{-5} pg of total RNA, and lane M: 100 bp DNA leader. (B) NASBA; Lane 1: 10^{5} pg, lane 2: 10^{4} pg, lane 3: 10^{3} pg, lane 4: 10^{2} pg, lane 5: 10^{1} pg, lane 6: 10 pg, lane 7: 1 pg of total RNA derived from infected cells, Lane M: DNA leader. (C) RT-LAMP; Lane 1: 10^{6} pg, lane 2: 10^{4} pg, lane 3: 10^{2} pg, lane 4: 10 pg, lane 5: 10^{-1} pg, lane 6: 10^{-3} pg and lane 7: 10^{-5} pg of total RNA, and lane M: 100 bp DNA leader.

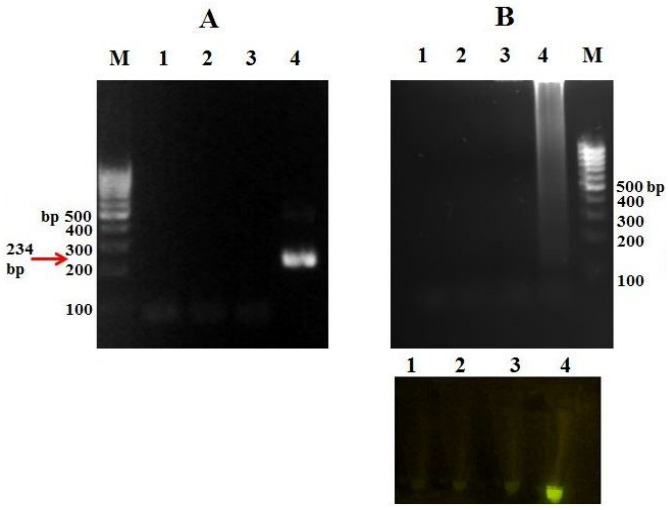

Fig. 4. The specificity of RT-PCR (A) and RT-LAMP (B) was determined using some serotypes of genus Enteroviruses. Lane 1: Coxsackievirus A16, lane 2: Echovirus 36, lane 3: Rhinovirus, lane 4: CVB3 and lane M: 100 bp DNA leader.

LAMP detection of CVB3 in cell culture. The results show that RT-LAMP is highest sensitive and suitable technique than RT-PCR and NASBA for detection of CVB3 infection. The sensitivity of these techniques was determined by serial dilution of total RNA extracted. This technique could detect viral RNA at the 100 fg of total RNA that extracted from CVB3-infected HeLa cells. Therefore, the sensitivity of RT-LAMP assay was 100 higher than RT-PCR and NASBA

Respectively. Also, RT-PCR and RT-LAMP method are very specific for detection of CVB3, so it can be used for clinical specimens. RT-LAMP technique, despite its simplicity, has a very high sensitivity and accuracy, and this method does not require expensive devices such as thermocycler, electrophoresis and gel dock, so this technique has been applied successfully for CVB3 detection.

CONCLUSIONS

Since CVB3 infections are associated with many important diseases in humans (such as type 1 diabetes and viral myocarditis), fast and accurate detection of people infected with this virus is very important. So far, different molecular methods such as RT-PCR, NASBA, and RT-LAMP have been used to detect this virus. The RT-LAMP method is very sensitive and selective method and it can be used as a preferred method for diagnosis due to its high speed and precision.

REFERENCES

[1] Y. Shen, Q.C. Kan, W. Xu, Y.W. Chu, S.D. Xiong, Iran. J. Allergy, Asthma and Immun. 8 (2009) 1.

[2] A.M. Feldman, D. McNamara, New Eng. J. Med. 343 (2000) 1388.

[3] G.W. Dec, Introduction to Clinical Myocarditis. Myocarditis. Humana Press, 2003.

[4] D. Fairweather, S. Frisancho-Kiss, S.A. Yusung, M.A. Barrett, S.E. Davis, R.A. Steele, S.J. Gatewood, N.R. Rose, J. Immun. 174 (2005) 261.

[5] L. Andréoletti, N. Lévêque, C. Boulagnon, C. Brasselet, P. Fornes, Archives of Cardiovascular Diseases 102 (2009) 559.

[6] M.L. Landry, R. Garner, D. Ferguson, J. Clin. Microbiol. 41 (2003) 346.

[7] K.N. Reetoo, S.A. Osman, S.J. Illavia, C.L. Cameron-Wilson, J.E. Banatvala, P. Muir, J. Gen. Virol. 81 (2000) 2755.

[8] D. Wang, X. Wang, Y. Geng, An C. Indian J. Med. Microbiol. 32 (2014) 124.

[9] U. Dierssen, F. Rehren, C. Henke-Gendo, G. Harste, A. Heim, J. Clin. Virol. 42 (2008) 58.

[10] L.M. Weiss, L.A. Movahed, M.E. Billingham, M.L. Cleary, Am. J. Pathol. 138 (1991) 497.

[11] J. Compton, Nature 350 (1991) 91.

[12] A. Saeedinia, M. Shamsara, M. Zeinoddini, V. Sadeghi, N. Maghsoudi, Iran. J. Biotechnol. 6 (2008) 222.

[13] B. Deiman, P. van Aarle, P. Sillekens, Mol. Biotechnol. 20 (2002) 163.

[14] J. Jean, B. Blais, A. Darveau, I. Fliss, Appl. Environ. Microbial. 67 (2001) 5593.

[15] T. Kievits, B. van Gemen, D. van Strijp, R. Schukkink, M. Dircks, H. Adriaanse, L. Malek, R. Sooknanan, P. Lens, J. Virological Methods 35 (1991) 273.

[16] J.C. Guatelli, K.M. Whitfield, D.Y. Kwoh, K.J. Barringer, D.D. Richman, T.R. Gingeras, Proceed. National Acad. Sci. 87 (1990) 1874.

[17] T. Notomi, H. Okayama, H. Masubuchi, T. Yonekawa, K. Watanabe, N. Amino, T. Hase, Nucl. Acids Res. 28 (2000) e63.

[18] L.M. Zanoli, G. Spoto, Biosensors 273 (2012) 18.

[19] L. Yan, J. Zhou, Y. Zheng, A.S. Gamson, B.T. Roembke, S. Nakayama, H.O. Sintim, Mol. Biosystems 10 (2014) 970.

[20] H. Zhang, K. Nie, Y. Liu, L. Luo, W. Huang, S. Zhou, M. Yang, Y. Chen, J. Luo, L. Gao, X. Ma, Adv. Infect. Diseases 2 (2012) 110.

[21] M. Arita, H. Ling, D. Yan, Y. Nishimura, H. Yoshida, T. Wakita, H. Shimizu, BMC Infect. Diseases 9 (2009) 208.

[22] K. Jaianand, P. Gunasekaran, M. Rajkumar, A.K. Sheriff, Int. J. Pharma and Biosci. 1 (2010) 03.

[23] K. Jaianand, N. Saravanan, P. Gunasekaran, A.K. Sheriff, Indian J. Med. Microbiol. 29 (2011) 110.

[24] W. Shi, K. Li, Y. Ji, Q. Jiang, M. Shi, Z. Mi, BMC Infect. Diseases 11 (2011) 197.

[25] K. Nie, S.X. Qi, Y. Zhang, L. Luo, Y. Xie, M.J. Yang, Y. Zhang, J. Li, H. Shen, Q. Li, X.J. Ma, PloS one 7 (2012) e52486.

[26] [26] H. Yaqing, Z. Wenping, Y. Zhiyi, W. Xionghu, Y. Shouyi, Y. Hong, D. Yingchun, H. Guifang, Lett. Appl. Microbial. 54 (2012) 233.

[27] G.P. Zhao, G.Y. Yin, H. Zhao, G.L. Li, B. Meng, X. Hu, Y.X. Li, Y.Z. Wang, H.B. Zhao, African J.

Microbiol. Res. 7 (2013) 4443.

[28] Q. Chen, Z. Hu, Zhang Q, M. Yu, J. Virolo. Mmethods 196 (2014) 139.

[29] X. Ding, K. Nie, L. Shi, Y. Zhang, L. Guan, D. Zhang, S. Qi, X. Ma, J. Clin. Microbial. 52 (2014) 1862.

[30] P. Jiang, Y. Liu, H.C. Ma, A.V. Paul, E. Wimmer, Microbiol. Mol. Biol. Rev. 78 (2014) 418.

[31] H.B. Zhao, G.Y. Yin, G.P. Zhao, A.H. Huang, J.H. Wang, S.F. Yang, H.S. Gao, W.J. Kang, Indian J. Microbial. 54 (2014) 80.

[32] T. Bourlet, S. Omar, F. Grattard, B. Pozzetto, Clin. Diagnostic Virol. 8 (1997) 143.

[33] R. Kandolf, Springer, Boston, MA, 1988, pp. 293-318.

[34] M. Hosoya, K. Honzumi, H. Suzuki, J. Infect. Diseases 175 (1997) 700.