DOI: https://doi.org/10.36719/2663-4619/89/320-328

Zarkhanim Aghayeva
Baku State University
zarxanim.agayeva@gmail.com

RAPID DIAGNOSTICS FOR HUMAN RHINOVIRUS C

Abstract

Human Rhinovirus C infection is associated with severe exacerbations in asthmatics and has also been linked to the development of asthma particularly in young children. To date, clinical diagnosis only informs us that Rhinovirus is the cause of infection but doesn't provide any information on the species type or etiological agent behind HRV C infections. Our research study aims to fill this research gap by developing a HRV C species specific rapid diagnostic test. To date, there is no rapid diagnostic method for HRV C identification owing to the high genetic variability within HRV C. In this project, we aimed at filling this gap by developing a novel diagnostic protocol targeting HRV C. For research, we used both conventional & Multiplex qPCR for detection of HRV C using different primer-probe combinations and optimizing reaction conditions like PCR annealing temperature and primer probe concentration. Our novel diagnostic RVC PCR was more specific for HRV C compared to the Gern PCR, as we obtained a 130 bp band specific for RVC in PCR gel electrophoresis. We also performed Real time PCR by optimizing the reaction conditions of our experiment.

Keywords: *human Rhinoviruses, rapid diagnostic method, PCR, respiratory diseases, Real time PCR*

Zərxanım Ağayeva
Bakı Dövlət Universiteti
zarxanim.agayeva@gmail.com

İnsanlarda Rinovirus C-in ekspress diaqnostikası

Xülasə

İnsanın Rinovirus C infeksiyası astmatiklərdə şiddətli kəskinləşmələrə və xüsusilə körpə uşaqlarda astmanın inkişaf etməsinə səbəb olur. Bu günə qədər kliniki diaqnostika yalnız rinovirusun infeksiyanın səbəbi olduğunu bildirir, lakin HRV C infeksiyalarına səbəb olan növ və ya etioloji agent haqqında heç bir məlumat vermir. Tədqiqat işimiz HRV C növünə xas sürətli diaqnostik test hazırlamaqla bu tədqiqat sahəsində boşluğu doldurmaq məqsədi daşıyır. Bu günə qədər HRV C yüksək genetik dəyişkənliyə malik olduğuna görə HRV C identifikasiyası üçün sürətli diaqnostik metod yoxdur. Tədqiqatlar zamanı biz HRV C-ni hədəfləyən yeni diaqnostik protokolu hazırlamaqla bu boşluğu doldurmağa çalışdıq. Tədqiqat üçün biz müxtəlif primer-zond kombinasiyalarından istifadə edərək HRV C-nin aşkarlanması üçün həm ənənəvi, həm də Multipleks qPCR-dən istifadə etdik və reaksiyanın şəraitini (PCR yumşalma temperaturu və primer zondun qatılığı) optimallaşdırdıq. Bizim yeni diaqnostik RVC PCR, Gern PCR ilə müqayisədə HRV C üçün daha spesifikdir, çünki biz PCR gel elektroforezində RVC üçün spesifik 130 bp diapazonu əldə edə bildik. Təcrübəmizin reaksiya şərtlərini optimallaşdırmaqla real vaxt rejimində PCR həyata keçirdik.

Açar sözlər: *insan Rinovirusu, ekspress diaqnostik üsul, PCR, tənəffüs xəstəlikləri, Real time PCR*

Introduction

Human Rhinoviruses (HRVs) are members of the family picornaviruses and genus enterovirus. HRVs are non-encompassed positive sense RNA virus. HRVs are the most predominant human respiratory pathogens which are liable for upper respiratory diseases known as the normal cold

(Jacobs et al., 2013). They thrive in similarly low temperature (33ºC) and this quality empowers them to colonize in the human nasopharynx. HRVs are significantly connected with serious lower respiratory ailments like pneumonia, bronchiolitis that frequently causes intensifications of asthma and chronic obstructive pulmonary diseases (COPD) (Lau et al., 2007; Winther, 2011). HRVs are the second most normal reasons for bronchiolitis in children after Respiratory Syncytial infection (RSV) and according to past investigations, early life diseases are regularly connected to the advancement of wheezing/asthma in new-born children (Miller et al., 2016; Liu et al., 2017; Vandini et al., 2019).

However, due to the high genetic variability within the HRVC strain and its genetic similarity to HRVA or B, it hinders the design of rapid HRVC diagnosis (Bochkov et al., 2012). If the diagnosis is available, antiviral drugs can be provided to people at increased risk of serious illness (those with HRVC infection) (Fuchs & Blaas, 2010; Costa, et al., 2014; Hammond et al., 2015; Erkkola, et al., 2020).

Materials and Methods

As RVC is genetically variable and there are over 50 types of RVC strains, it is time intensive to design primer for each. So, our primer mix targeted all the 50 strain types. 35 Primers were used in our study compared to 4 primers used in the Gern PCR (Bochkov et al., 2014) & 2 primers used in standard PCR. We performed a primer concentration optimization PCR to get the maximum output. Gradient used was (1-10µm concentration). We only got bands for primer concentration 10µm and 5 µm (Figure 1). 10 µm is the standard concentration for a normal PCR, with two primers this is doubled, where as in our assay we used 35 primes so it was 17.5x more primers used in our assay.

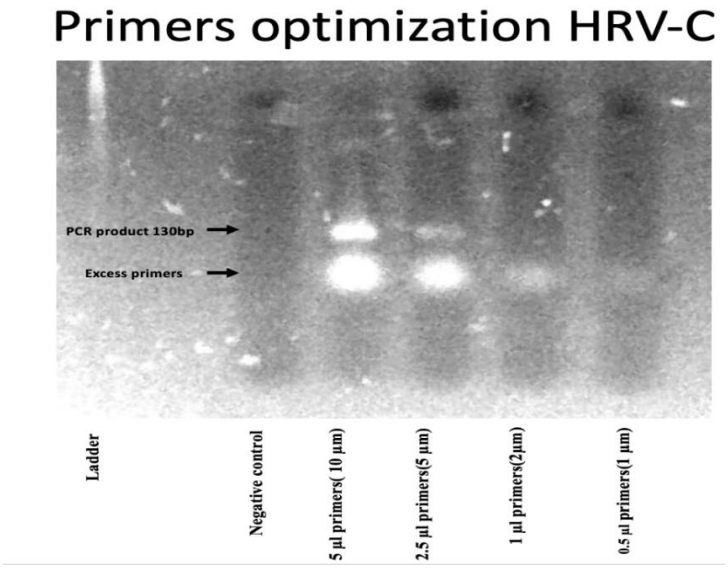

Figure 1. Picture representative of gel doc image of amplified PCR products (Using different primer concentrations). From left Lane: Lane 1-100 bp DNA hyper ladder, followed by Lane 3 to Lane 6 with PCR products obtained at different primer concentration in decreasing order i.e., 10 µm 5µm, 2µm & 1µm respectively. Lane 2 refers to negative control where no band was seen.

Real time PCR trial with HRVC specific TaqMan FAM probe

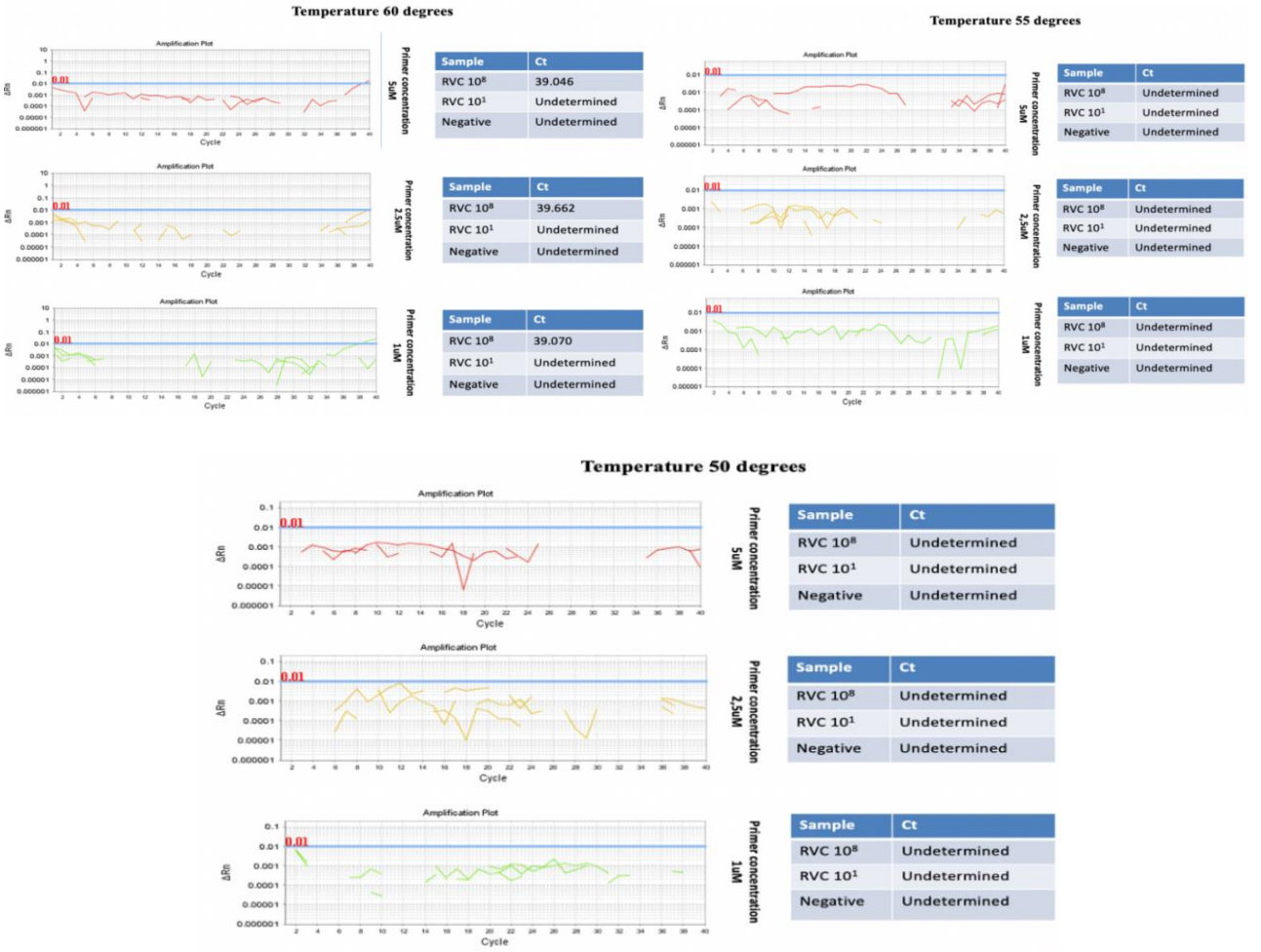

Figure 2. Real time PCR was performed testing the novel HRVC specific primers and probe. PCR was performed using a high and low concentration of DNA template (HRVC plasmid high 10^8 and low concentration 10^1). PCR was performed at varying temperatures (60, 55 & 50ºC) and with varying primer concentrations (5ul (10um), 2.5ul (5um), 1ul (2um)). Graphs show the amplification curves of RT-PCR with threshold values set at 0.01. Tables show the corresponding Cycle threshold (CT) values for each PCR condition. PCR analysis was performed in. Step One Plus™ v2.3.

In our experiment, FAM refers to the fluorescent dye on the probe which binds to our target DNA to give us amplified product after each cycle. This PCR helps to monitor the progress of our reaction in real time unlike conventional gel PCR where the amplified product is detected at the very end of PCR reaction using a DNA gel. At the end of the 60-degree PCR showed amplification of 39 which can be attributed to low viral load as we know that a higher Ct value refers to low viral load and Ct values are inversely proportional to the viral load in the sample (Figure 2). We initially tested the PCR using the recommended conditions that came with the gene expression master mix. This type of PCR is generally more sensitive that standard PCR, more specific and is routinely used in diagnostic labs for detection of infectious agents. Currently used to detect if RV is infectious agent.

We used a mixture of probes as well 12 probes to perform a TaqMan PCR. But no copies of viral RVC was detected. Ct value gives us information about the total amount of viral genetic material present in the clinical sample. Here we couldn't infer anything as none of the lines except the PCR at 60 degrees crossed the threshold 0.01 set.

SYBR Green PCR

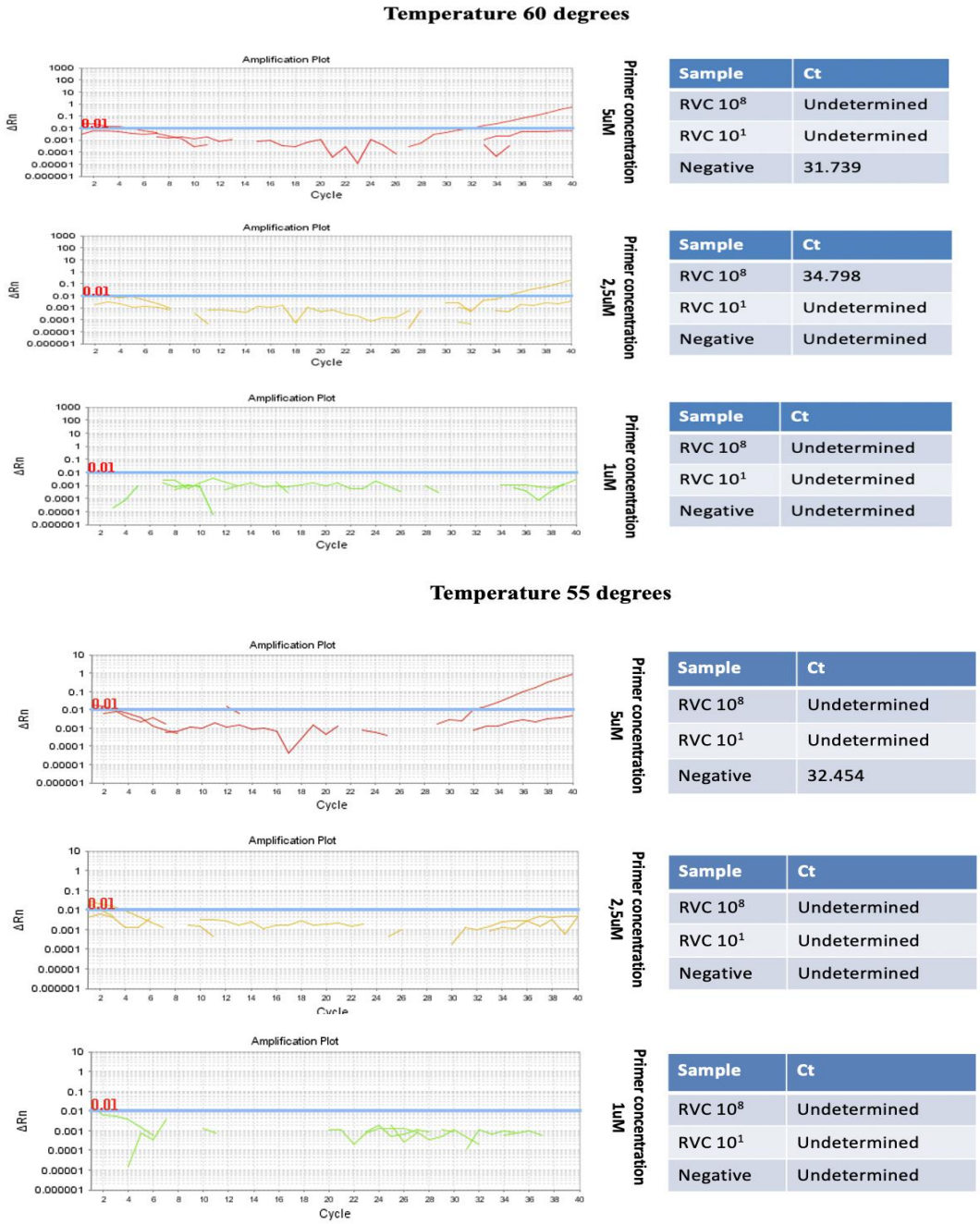

Figure 3. Real time PCR was performed testing the novel HRVC specific primers PCR was performed using a high and low concentration of DNA template (HRVC plasmid high 10^8 and low concentration 10^1). PCR was performed at varying temperatures (60 & 55) and with varying primer concentrations (5ul (10um), 2.5ul (5um), 1ul (2um). Graphs show the amplification curves of RT-PCR with threshold values set at 0.01. Tables show the corresponding Cycle threshold (CT) values for each PCR condition. PCR analysis was performed in. Step One Plus™ v2.3.

As the previous experiments with the probe didn't work, we were assuming that there was a problem with the probe used. So, we conducted a SYBR Green PCR using 35 primers instead along with the fluorescent dye SYBR green (Figure 3). SYBR Green is a dsDNA binding dye intercalating nonspecifically into dsDNA. This allows measurement of the amount of PCR product produced during PCR (DNA). SYBR green fluorescence increases as the amplification proceeds & the amount of DNA product increases. The fluorescence intensity is directly proportional to the

amount of product accumulated or the number of SYBR green molecules incorporated into dsDNA. In qPCR the number of cycles required to reach the threshold level is calculated with the help of a C_t or Cq value. SYBR green is a fluorescent dye that is sensitive to dsDNA as it encounters more dsDNA in the sample it binds and produces more fluorescence. If this experiment worked it would mean that there was a problem with the probe used in previous experimental designs. A Ct value was only observed for the 60-degree FAM trial PCR as seen from the amplification curve, but the Ct value was very high i.e., 39 which meant that the viral load was too low or negligible. Whereas the other melting curves of the remaining experiment SYBR Green PCR and TaqMan PCR did not produce any amplification and the Ct values were undetermined (Figure 4).

Also, for the SYBR green & TaqMan PCR experiments we did not use the 50º C temperature for the PCR, as it did not seem to work in the initial experiment, so we decided to opt it out and hence did not check the primer efficiency at this low temperature for this and further experiment.

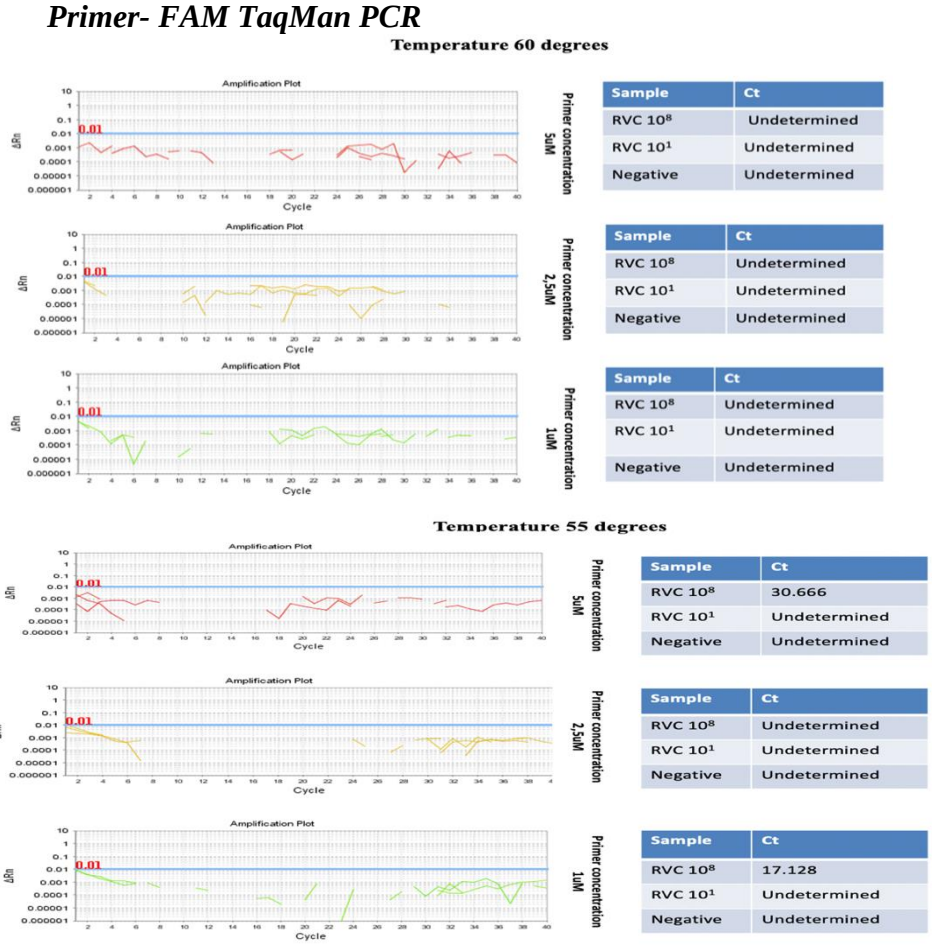

Figure 4: Real time PCR was performed testing the novel HRVC specific primers and probe. PCR was performed using a high and low concentration of DNA template (HRVC plasmid high 10^8 and low concentration 10^1). PCR was performed at varying temperatures (60 & 55) and with varying primer concentrations (5ul (10um), 2.5ul (5um), 1ul (2um). Graphs show the amplification curves of RT-PCR with threshold values set at 0.01. Tables show the corresponding Cycle threshold (CT) values for each PCR condition. PCR analysis was performed in. Step One Plus™ v2.3.

Results and Discussion

The emergence of molecular virus diagnosis has broadened our understanding of the epidemiology of respiratory diseases by increasing the detection rate for known viruses. These new diagnostic techniques are particularly useful for understanding the role of HRV, which is particularly difficult to cultivate in tissue culture, (Iwane et al., 2011). The development of

comparable tests for HRV has been affected by its greater genetic variability and paucity of substantial published HRV data sequences (Sikazwe et al., 2011). To date, there is no rapid diagnostic method for HRV C identification owing to the high genetic variability within HRV In this context, we used both conventional & Multiplex qPCR in our study for detection of HRV C with the eventual intention of developing a novel diagnostic assay targeting HRV C.

Multiplex qPCR assays have made important contributions to the rapid identification of viruses linked with acute respiratory infections in recent years, enabling for the rapid implementation of interventions and preventive strategies to limit the spread of diseases with high transmissibility.

Dotsch et al. (2001) developed a multiplex qPCR assay that could simultaneously measure 16 different types of Human Adenovirus (HAdV) serotypes that cause respiratory tract infections. The multiplex qPCR used in his work could not only detect the three HAdV species (HAdV B, C, and E) simultaneously and precisely, but it also had a high sensitivity (100 percent). The multiplex qPCR proved highly specific for the target gene (Dotsch et al., 2001).

The multiplex qPCR was also highly specific for three different viral species, with no mutual cross-reactivity. This study revealed that multiplex qPCR had a higher sensitivity than the immunofluorescence techniques. In comparison to typical cell culture, the entire experiment took only 1.5 hours from nucleic acid extraction to qPCR results.

This could not only help to make an accurate diagnosis based on the qPCR result in a timely manner, but it could also help to increase its efficiency.

During qPCR, each amplicon amplifies at the same time and is detected using separate fluorescence spectra.

TaqMan detection is very sensitive and can detect very low levels of genes (as low as 10 to 100 copies), so compared to traditional RNA detection, a very small amount of mRNA is required (nanogram level) (Gangisetty and Reddy, 2009).

Fluorescent based PCR is a "closed tube" system that avoids time-consuming and hazardous post-PCR processing and reduces potential contamination risks. These PCRs enables accurate and reproducible quantification because it is based on Ct values rather than endpoint detection, where the PCR component limits the rate (Gangisetty and Reddy, 2009).

In real-time PCR analysis, at any number of cycles, there is a quantitative relationship between the number of input target templates and the amount of PCR products. In Real time PCR, purified PCR products generated using specific primers for each target are used as templates to generate a standard curve. In this study, we have used plasmids as template DNA and although we had a standard curve, we only used the 10^1 to 10^8 samples to optimize the PCR so, a standard curve of HRV C DNA (10^1-10^8 copies of template cDNA) was constructed (Sikazwe et al., 2016).

Although the use of PCR for viral quantification is common, a number of limitations may result in inaccurate quantification, especially in two step PCRs. The one-step real-time RT-PCR is a welcome simplification of the two-step RT-PCR, saving time, and resulting in a lower risk of technical errors. In the Two- step PCR cDNA synthesis followed by RT PCR is performed using patient's samples. Where as in one step PCR normally cDNA synthesis and PCR reaction is performed in one tube. In this project, we performed the two step PCR that bypasses Reverse transcription step and uses plasmids specific for HRV C as template DNA instead.

According to reports, the reproducibility of the one-step assay is higher, implying that the quantification reliability is better in this format (Schibler et al., 2012).

However, there are only a few published PCR tests that combine reverse transcription and PCR in the same real-time reaction (i.e., one-step test). e. (Liu et al., 2017; Roussy et al., 2014; Schibler et al., 2012). Advantages of the one-step method used in our study over the two-step method include improved workflow, reduced test set-up time, and elimination of cross-contamination during the cDNA transfer process from the reverse transcription reaction to PCR reaction. One-step analysis methods have been developed for a limited number of PCR amplification platforms and chemicals, and may not necessarily work optimally with other platforms.

The high PCR efficiency of this platform generally requires the use of TaqMan primers and probes (Applied Biosystems). The high PCR efficiency of this platform generally requires the use of TaqMan primers and probes (Applied Biosystems). The melting temperature is 58°C to 60°C and 68°C to 70°C, and the size of the amplicon is 50°C to 150. bp. The five noncoding regions of the rhinovirus genome are the most common targets for PCR detection (Kieninger et al., 2013), consisting of 6 subregions (designated A to F) of approximately 20 bases, these subregions are highly conserved in Picornavirus and separated by longer intermediate variable sequences (Sikazwe et al., 2016). These features complicate the design of an efficient one-step real-time analysis. In this step, real-time PCR detection, reverse transcription, and PCR amplification are performed in the same reaction tube. In this project we have performed a real time analysis utilizing multiple primers and probes in a single reaction tube.

If the amplification curve of the repeated reaction (i.e. the change in the standardized report signal [delta Rn] and the number of PCR cycles) shows an exponential increase in the fluorescence signal, the sample is considered positive. In addition, most of the real-time analysis for rhinovirus detection is a two-step method that requires manual transfer of cDNA in the PCR reaction. Therefore, it is more troublesome than single-stage PCR detection and more susceptible to contamination.

We also performed quantitative Real time TaqMan PCR, for testing the probe (FAM) but the qPCR Ct values suggested that the results were negative as no HRV C Viral DNA was detected. So here we couldn't infer anything as none of the lines except the one at 60 degrees crossed the threshold 0.01 set. Although the Ct value could be assigned for certain samples, it was a very late Ct which wouldn't be promising to be utilized in a rapid diagnostic and also wasn't sensitive as this was with high copy number samples 10^8 plasmid copies which is a very high density. Therefore, we switched to SYBR Green PCR and used a mixture of 35 primers designed by Dr Jenny Herbert, University of Manchester, to see if there was underlying problem with the probe used. If this experiment worked it would mean that our probe was not efficient. But it was otherwise, the result of this experiment was negative as well which meant that the primers were inefficient. This could be attributed to the multiplex nature of our experiment, where a mixture of 35 primers used in one tube, was very likely to inhibit the progress of our PCR reaction. So, in the next experiment we again opted for the TaqMan PCR using probe and fewer primers i.e., TaqMan PCR to see whether the probe would amplify when fewer primer pairs were used in the reaction instead.

We had to use primer 1 for the R and F primers as these covered the RVC strain 15 genomes which was present in the plasmid. So, although still high with 20 primers in the mix, this was still significantly reduced from the 35primers used in the original PCR. Still no viral HRV C DNA was detected. So, our experiment failed yet again. Our experiments clearly reveals that our primers and probes were simply not efficient for developing a novel rapid diagnostic test. This could be attributed to a number of reasons such as Single Nucleotide Polymorphisms in Primer/ probe target region. According to a recent study by (Randhawa et al., 2011) it was stated that Multiple mismatches in the probe target region may have a higher influence on accuracy than a single mismatch, for accurate diagnosis of virus in the sample. Primers and probes must be constructed to match the target sequence in order to accurately quantify viral load in clinical samples, to yield the highest fluorescence signal possible

The experiments could have been repeated with 1 primer pair and probe but owing to time constraint in this thesis we couldn't perform that experiment however our approach is novel and can be exploited for future research work.

Conclusion

To achieve the goal of our project, we used a mixture of probes and primers, and optimized reaction conditions, such as changes in primers, probe concentration, and hybridization temperature, to develop a new diagnostic test for the detection of RVC. Our research was novel as a multiplex PCR was performed utilizing 35 primers in one single tube targeting all 50 strains of HRVC, an

approach that has largely remained unexplored so far as no research publications has been found pertaining to this. Our study would be useful for updating of diagnostic assays for accurate comprehensive detection of circulating RV's. Changing the primer sequences may help and further optimization needs to be performed. Expanding routine testing for these viruses will help to better define the epidemiology of HRV infection and the extent and burden of HRV disease.

References

1. Jacobs, S. E., Lamson, D. M., St George, K. & Walsh, T. J. (2013). 'Human rhinoviruses' Clin Microbiol Rev, 26 (1), pp.135-62.
2. Lau, S.K., Yip, C.C., Woo, P.C. & Yuen, K.Y. (2010). 'Human rhinovirus c: A newly discovered human rhinovirus species' Emerg Health Threats J, 3 p.e2.
3. Winther, B. (2011). 'Rhinovirus infections in the upper airway' Proc Am Thorac Soc, 8 (1), pp.79-89.
4. Miller, E.K., et al. (2016). 'Hospitalizations and outpatient visits for rhinovirus-associated acute respiratory illness in adults' J Allergy Clin Immunol, 137 (3), pp.734-43 e1.
5. Liu, L., et al. (2017). 'Association between rhinovirus wheezing illness and the development of childhood asthma: A meta-analysis' BMJ Open, 7 (4), p.e013034.
6. Vandini, S., Biagi, C., Fischer, M. & Lanari, M. (2019). 'Impact of rhinovirus infections in children' 11 (6), p.521.
7. Bochkov, Y.A. et al. (2012). 'Clinical and molecular features of human rhinovirus c' Microbes Infect, 14 (6), pp.485-94.
8. Fuchs, R. & Blaas, D. (2010). 'Uncoating of human rhinoviruses' Rev Med Virol, 20 (5), pp.281-97.
9. Costa, L.F., et al. (2014). 'Human rhinovirus and disease severity in children' Pediatrics, 133 (2), pp.e312-21.
10. Hammond, C., Kurten, M. & Kennedy, J.L. (2015). 'Rhinovirus and asthma: A storied history of incompatibility' Curr Allergy Asthma Rep, 15 (2), p.502.
11. Erkkola, R., et al. (2020). 'Rhinovirus c is associated with severe wheezing and febrile respiratory illness in young children' Pediatr Infect Dis J, 39 (4), pp.283-286.
12. Bochkov, Y.A., et al. (2014). 'Improved molecular typing assay for rhinovirus species a, b, and c' J Clin Microbiol, 52 (7), pp.2461-71.
13. Iwane, M.K., Prill, M.M., Lu, X., Miller, E.K., Edwards, K.M., Hall, C.B., Griffin, M.R., Staat, M.A., Anderson, L.J., Williams, J.V. and Weinberg, G.A., 2011. Human rhinovirus species associated with hospitalizations for acute respiratory illness in young US children. Journal of Infectious Diseases, 204(11), pp.1702-1710.
14. Sikazwe, C.T., Chidlow, G.R., Imrie, A. and Smith, D.W., 2016. Reliable quantification of rhinovirus species C using real-time PCR. Journal of virological methods, 235, pp.65-72.
15. Dotsch, J., Repp, R., Rascher, W. & Christiansen, H. (2001). 'Diagnostic and scientific applications of taqman real-time PCR in neuroblastomas' Expert Rev Mol Diagn, 1 (2), pp.233-8.
16. Gangisetty, O. and Reddy, D.S., 2009. The optimization of TaqMan real-time RT-PCR assay for transcriptional profiling of GABA-A receptor subunit plasticity. Journal of neuroscience methods, 181(1), pp.58-66.
17. Schibler, M., Yerly, S., Vieille, G., Docquier, M., Turin, L., Kaiser, L., Tapparel, C., 2012. Critical analysis of rhinovirus RNA load quantification by real-time reverse transcription-PCR. J. Clin. Microbiol. 50, pp.2868–2872
18. Lee, W.M., et al. (2007). 'A diverse group of previously unrecognized human rhinoviruses are common causes of respiratory illnesses in infants' PLoS One, 2 (10), p.e966.
19. Roussy, J.-F., Carbonneau, J., Ouakki, M., Papenburg, J., -È. Hamelin, M., De Serres, G., Boivin, G., 2014. Human metapneumovirus viral load is an important risk factor for disease severity in young children. J. Clin. Virol. 60 (2), pp.133–140.

20. Kieninger, E., et al. (2013). 'Rhinovirus infections in infancy and early childhood' Eur Respir J, 41 (2), pp.443-52.
21. Randhawa, P., Kant, J., Shapiro, R., Tan, H., Basu, A. and Luo, C., 2011. Impact of genomic sequence variability on quantitative PCR assays for diagnosis of polyomavirus BK infection. Journal of clinical microbiology, 49(12), pp.4072-4076.

Received: 26.01.2023 Accepted: 10.03.2023