JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Coronavirus Pandemic

Development of multiplex immuno-PCR diagnostic platform using chicken IgY antibodies for COVID-19 diagnosis

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Abstract

Introduction: The coronavirus disease 2019 (COVID-19) pandemic has significantly accelerated the development of diagnostic techniques. Real-time quantitative polymerase chain reaction (RT-qPCR) was the method of choice for diagnosis and was considered as the gold standard. However, limited specificity of RT-PCR was noticed during the pandemic. This research aimed to develop a combined highly specific immune-based and highly sensitive molecular-based diagnostic technique.

Methodology: Groups of chicken were immunized with commercial severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) N, S, and E antigens. The IgY antibodies were purified from eggs using a High-Trap IgY affinity column. Three unique DNA barcodes were designed, synthesized, and amplified using 5′-amine–labeled forward primers. DNA barcodes purified form PCR products were coupled to IgY antibodies using the (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) - N-hydroxysuccinimide (EDC-NHS) coupling chemistry. ELISA; SDS-PAGEs; immunoblot (IB); and uniplex-, duplex- and multiplex immuno-PCR (IPCR) were used to confirm system validity.

Results: Amplification of single barcodes using RT-PCR showed a Ct value of 15, with no significant variation when amplified in duplex or multiplex formats. Chicken IgY–DNA barcode conjugation and reactivity were verified using IB and ELISA. IPCR resulted in efficient amplification of all three DNA barcodes in uniplex, duplex, and multiplex formats after binding to commercial N, S, and E antigens.

Conclusions: The successful combination of the specific antibody-based techniques, low-cost chicken IgY antibodies, and RT-PCR sensitivity achieved in this study present a promising approach to meet the demand for sensitive and accurate diagnostics. This generic platform can be adopted in any analyte detection system.

Key words: SARS-CoV-2, IgY; barcodes; RT-PCR; IPCR; multiplex-IPCR.

J Infect Dev Ctries 20205; 19(10):1455-1463. doi:10.3855/jidc.21414

(Received 03 February 2025 – Accepted 24 April 2025)

Introduction

Despite the devastating impact of coronavirus disease 2019 (COVID-19) on all aspects of human life, it has since been largely neglected by policymakers—though not by researchers. The betacoronavirus designated as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent of COVID-19 [1]. SARS-CoV-2 infection can transmit rapidly from person-to-person. According to the World Health Organization (WHO), SARS-CoV-2 has led to over 774 million confirmed cases worldwide and more than 7 million deaths as of 2024 [2,3].

COVID-19 was not the first pandemic to threaten human life. The world has endured many endemics and outbreaks throughout history [4]. Early and accurate diagnosis is mandatory for the effective containment of such pandemics. The heterogeneity of disease manifestation in COVID-19 ranges from non-symptomatic to severe, and the diversity of non-specific clinical signs reinforce the need for complementary tests with high sensitivity and specificity [5].

The 30 Kb positive-strand RNA genome of SARS-CoV-2 encodes four essential structural proteins and several smaller “accessory” proteins. These include the spike (S) protein, nucleocapsid (N) protein, membrane (M) protein, and envelope (E) protein; all of which are required to produce a structurally complete viral particle. While each protein primarily contributes to the virus particle's structure, they are also involved in other aspects of the replication cycle [6,7].

Reverse transcription real-time polymerase chain reaction (rtRT-PCR) is considered the gold standard technique for disease diagnosis [8,9]. Although diagnostic kit manufacturers have invested time and money to overcome most of the problems associated with nucleic acid detection by rtRT-PCR, some limitations still exist. These include the need for well-trained personnel; the need for high-tech equipment [10]; and the challenges related to probe-based diagnostics, such as mutations in the virus

genome that can render probes and primers ineffective; leading to false-negative results [11–13].

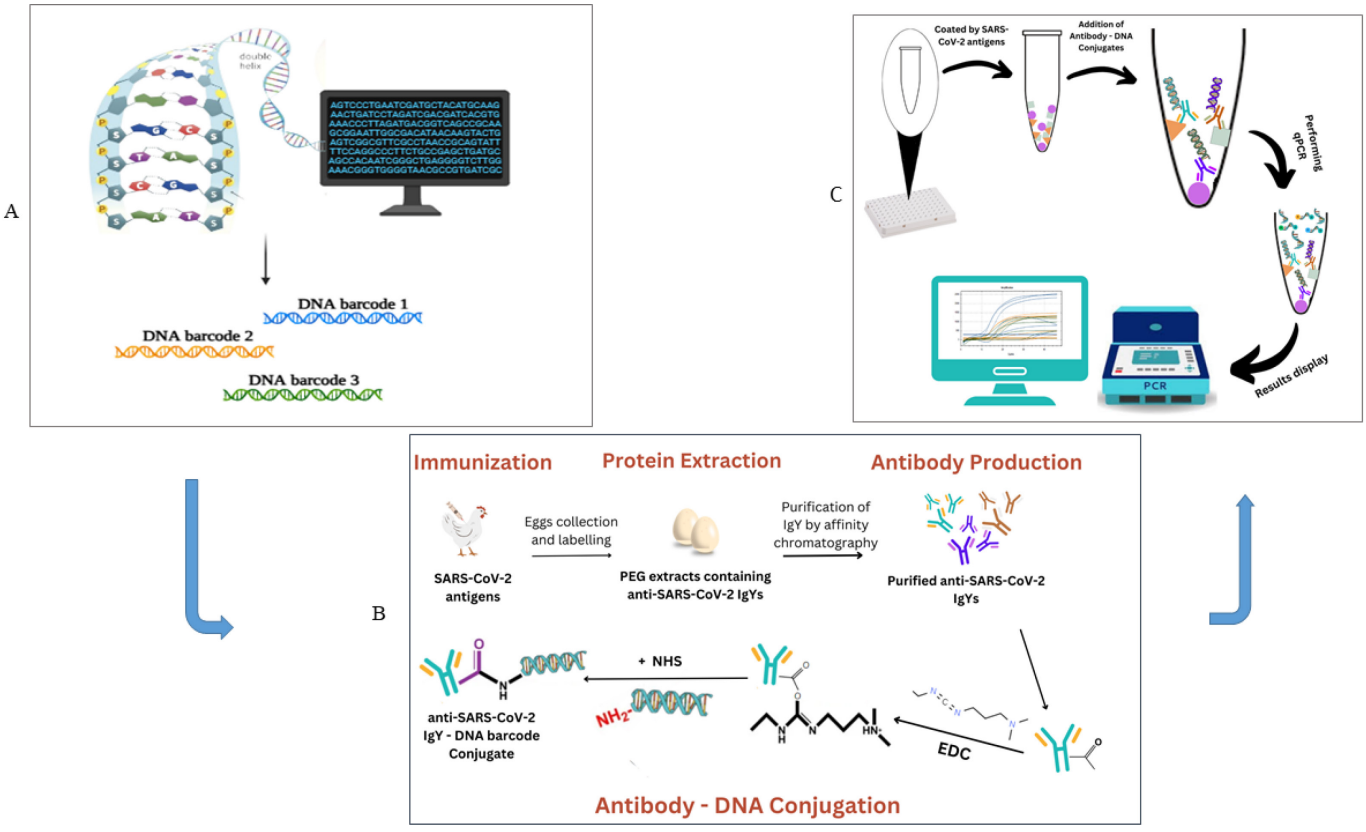
Rapid tests that detect viral antigens present in nasopharyngeal swabs have been introduced to overcome the need for sophisticated, time-consuming rtRT-PCR [10]. This technique is based on the detection of viral proteins using specific antibodies. The efficacy of these rapid tests varies [14]. The quality and type of antibodies, i.e., polyclonal or monoclonal, are the main considerations in ensuring high efficacy for such tests [15].

Immunoglobulins (antibodies) that are secreted by plasma cells against a particular antigen vary depending on the required immune response. IgG is one of the abundant types of antibodies secreted in the blood of chicken. It is referred to as IgY when transferred to the egg yolk. IgY obtained from egg is easier to acquire and more abundant compared to other antibodies found in the chicken serum [16]. However, tests have shown limited sensitivity and a low rate of detecting antigens, such as SARS-CoV-2 antigens. [17].

A typical polymerase chain reaction (PCR) can detect a single molecule of RNA in a microliter of solution, whereas antigen tests require a sample to contain thousands to tens of thousands of virus particles per microliter to produce a positive result [18]. The Centers for Disease Control and Prevention (CDC) and WHO did not recommend the use of these tests for COVID-19 diagnosis due to their low sensitivity, even though hospitals applied them as a screening tool for patients visiting clinics or requiring admission, and the results may have been used to inform public policies [19]. It can be concluded that the molecular techniques with remarkable sensitivity may lack sufficient specificity, while the antibody-based systems offer high specificity but may lack sensitivity. Therefore, a combination of both techniques may offer a significant solution for SARS-CoV-2 diagnosis.

Immuno-PCR (IPCR) is a technique that provides a combination of highly specific antibody-based immunoassay and highly sensitive PCR. It may reach 102–104-fold detection sensitivity in comparison to the

Figure 1. Illustration of the three phases of multiplex IPCR development. **A:** Generation of the three DNA barcodes using bioinformatics tools. **B:** Anti-SARS-CoV-2 N, S and E IgY antibodies production, purification, and antibody-DNA conjugation protocol using the chemical reaction of carbodiimide-based approach. C, the multiplex IPCR protocol starting with microplate coating with recombinant antigens, addition of IgY-DNA conjugates, amplification, and analysis.



IPCR: immune-polymerase chain reaction; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; PEG: polyethylene glycol; qPCR: quantitative polymerase chain reaction; N: nucleocapsid; S: spike; E: envelope.

Table 1. Sequences of the DNA barcodes used as templates for the real-time polymerase chain reaction (RT-PCR) amplification.

DNA barcode	Sequence
DNA barcode 1	5'-CAC TAT CAA GAC AAT CAC AAC TCA GCC TCG GAA TCG TAC TGA TCC TAA CTG TAA CAT ACT ACA CGT CGA AAT ATC AAT CTA GTA GGA TTT GCG TTC AGT ACA GGA GCG AGA CGT ATA TCG CAC AGC ATA TCC CTA TCA TGA CCG CAT AGC-3'
DNA barcode 2	5'-GGT AGA TGA GTC TAG AGG ATC AGA TAT GGC CGA CAG CTT GGA ACA GAC TAA AGA CCG AAT GAG ACA GTA CAC AAT TTC CAG TCA TCC GAG CTG ACT GAC GAT CCT ATC GTG CAA ATC AGT CGA TCC CTA CAT CGG TGT AGC TCA AAG TCG-3'
DNA barcode 3	5'-AAT TCC GGT TCT AGT CAT CAG TCA GTC AGG TTC CAA GCT ACG TAC GCT ACG TAC GTC ACG TAA ACG GGG TCC AGC TAC GAT CGA TCG ATC GAT CGA TCG ATC TAG CGA CGA TCG AAT ATG GCT AGC TAG TCG ATC GAT CGA TCG ATC AAC-3'

analogous enzyme linked immunosorbent assay (ELISA). This technique has been utilized for the detection of analytes in minute quantities in blood, such as cancer markers, hormones or cytokines [20–22]. It has been documented that it could detect as little as 0.5 pg of hepatitis B surface antigen (HBsAg) in a serum sample [23]. This study aimed to develop a multiplex IPCR diagnostic technique using chicken IgY, as a combination of specific immunoassay and sensitive molecular techniques for COVID-19 diagnosis, which would offer a robust diagnostic method. Multiple antigens, namely S, N, and E, were targeted simultaneously to improve the diagnostic efficacy.

Methodology

Generation of unique DNA barcodes and primers

Three unique DNA barcodes (DNA-1, DNA-2, and DNA-3) (Figure 1A, Table 1) were designed manually using the National Center for Biotechnology Information (NCBI) software by inserting 150 randomly ordered nucleotides. They were modified several times to get the required sequences based on several criteria, including self-dimerization, hetero-dimerization, and ΔG (≥ – 10). The Integrated DNA Technology (IDT) software was used to perform this analysis. Moreover, melting temperature, length, complementary sequence of the generated single stranded DNA, CG contents, and molecular weight were taken into consideration for the purpose of characterization. Primers and amine-modified (5' amino modifier C6 (/5AmMC6/))-forward primers (NH2-IPCR- F) (Table 2) were designed and synthesized using IDT software tools OligoAnalyser and PrimerQuest. Furthermore, the NCBI software was used to assess the similarity level with other genetic materials related to any microorganisms that could harbor in the nasopharyngeal region of the human respiratory system. The probes were designed using IDT software (Table 2) and synthesized by Microsynth AG, (Balgach, Switzerland). The fluorophores FAM, HEX, and ROX were conjugated to DNA-1, DNA-2, and DNA-3, respectively. Each DNA barcode was synthesized as a pUC57 plasmid insert by GenScript Biotech Inc (Piscataway, USA). The plasmids were transformed into *E. coli* competent cells to have a sustained DNA barcode source. The DNA barcodes and their primers sets (primers and probes) were tested and optimized using conventional and RT-PCR.

Amplification and purification of DNA barcodes

DNA barcodes were amplified by adding 1X of 2X Promega Go *Taq*™ Master Mixes (Promega Co., Madison, USA), 0.5 nM of either amine-modified forward primer for antibody conjugation or non-modified forward primer for quantification by RT-PCR, 0.5 nM of reverse primer, 0.2 nM of the template plasmid, and nuclease free water (NFW) up to a final volume of 20 µL. The PCR amplification program used was 95 ºC for 3 min as the initial denaturation step; 35 cycles at 95 ºC for 30 sec, 60 ºC annealing temperature

Table 2. The forward and reverse primers, amine-modified forward primers, and probe sequences and their fluorescence labeling for each DNA barcode.

DNA barcodes	Primer/probe name	Primers/probes sequences
DNA barcode 1	IPCR-1 F	5` CTCGGAATCGTACTGATCCTAAC 3`
	IPCR-1 R	5` ATGCGGTCATGATAGGGATATG 3`
	NH2-IPCR-1 F	5`AmMC6/CT CGG AAT CGT ACT GAT CCT AAC 3`
	Probe 1	FAM-TTTGCGTTCAGTACAGGAGCGAGA- BHQ-1
DNA barcode 2	IPCR-2 F	5` CGACAGCTTGGAACAGACTAAA 3`
	IPCR-2 R	5` CGATGTAGGGATCGACTGATTTG 3`
	NH2-IPCR-2 F	5` AmMC6/CG ACA GCT TGG AAC AGA CTA AA 3`
	Probe 2	HEX-TCATCCGAGCTGACTGACGATCCT-BHQ-1
DNA barcode 3	IPCR-3 F	5` CGGTTCTAGTCATCAGTCAGTC 3`
	IPCR-3 R	5` GCCATATTCGATCGTCGCTA 3`
	NH2-IPCR-3 F	5` AmMC6/CG GTT CTA GTC ATC AGT CAG TC 3`
	Probe 3	ROX-ACGCTACGTACGTCACGTAAACGA-BHQ-2

IPCR: immune-polymerase chain reaction.

for 30 sec, and 72 ºC extension for 15 sec 58°C; and 3 min of final extension at 72 ºC. The PCR was performed using the ESCO PCR thermal cycler (Taichung, Taiwan).

The DNA barcodes were recovered from the PCR amplification product by the isopropanol precipitation method. This was performed by adding 20 µL of 3M sodium acetate (Fisher Scientific, Waltham, USA) at pH 5.2 to 200 µL of the PCR product of the intended DNA barcode. 220 µL of isopropanol (Tedia, Fairfield, USA) was added, and then centrifuged at 15000 x g at 4 °C for 30 min. The resultant supernatant was discarded, and 600 µL of cooled 70% ethanol was added and centrifuged once again. The supernatant was discarded carefully, and the pellet was left to air dry. After that, the pellet was dissolved in 50 µL of TE buffer (pH 8.4), and the concentration was measured using a Thermo Scientific™ µDrop plate (ThermoFisher Scientific, Waltham, USA) and stored at – 20 °C for later use in antibody-DNA conjugation.

Quantification of DNA barcodes using RT-PCR

*Taq*Man–based RT-PCR was optimized to quantify the DNA barcodes. The reaction was performed using 10 µL of Premix Ex *Taq*™ (probe RT-PCR), bulk (2X) master mix (Takara Bio Inc, Kusatsu, Japan), 0.5 nM of both forward and reverse primers, 0.5 nM of probe ((P1 (FAM) / P2 (HEX) / P3 (ROX)) or all of them in case of multiplexing), 0.2 nM of DNA barcode, and NFW was added to reach a final volume of 20 µL. The reaction underwent an initial denaturation step at 95 °C for 3 minutes; followed by 45 cycles of 95 ºC for 30 seconds, 60 ºC annealing for 30 seconds, and 72 ºC extension for 15 seconds.

Chicken immunization and anti-SARS-CoV-2 S, N, and E IgY production, purification, and characterization

Chicken accommodation, immunization, and blood collection were done according to Al-Qaoud *et al*. [24] after obtaining approvals from the Yarmouk University Animal Care and Use Committee (No. AICUC/2021/11). Twenty-week-old Novogen White (Longhorn) egg layer hens were immunized intramuscularly in the chest with SARS-CoV-2 proteins containing 400 µg mixed 1:1 with complete Freund's adjuvant (CFA) (Thermo Fisher, Waltham, USA). The boosters were repeated 3 times, 2 weeks apart, after mixing the antigens with incomplete Freund's adjuvant. Recombinant N, S, and E viral proteins of SARS-CoV-2 were purchased from Ray Biotech Inc. (Peachtree Corners, USA). The blood samples were collected from chicken in plain vacutainer tubes (FL Medical, Torreglia, Italy) and left to clot. Serum was collected after centrifugation of tubes at 2000 × g (MPW-251, Warsaw, Poland). Sandwich ELISA was performed to measure antigen specific IgY in serum and egg extract, as described elsewhere [24]. IgY antibodies were extracted from chicken yolk using polyethylene glycol (PEG) and purified with a HiTrap IgY purification column (Cytiva, Uppsala, Sweden).

Conjugation of DNA barcodes to antibodies

DNA barcodes were amplified using amine-modified forward primers. The PCR product was dialyzed and concentrated to reach the required concentration in purified form to be then conjugated with the corresponding anti-SARS-CoV-2 IgY antibodies. Carbodiimide ((1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)/N-hydroxysuccinimide (NHS))-based chemistry was used for antibody-DNA conjugation (Figure 1B). Approximately 10 mg/mL of the purified anti-SARS-CoV-2 IgY dissolved in 1 mL of activation buffer [0.1M of MES + 0.5M of NaCl, pH 6] was obtained using 30 kDa MWCO Vivaspin® 2 HY Centrifugal Concentrator, Cytiva (Uppsala, USA). After that, 1.4 mg of EDC (Sigma Aldrich Inc., St. Louis, USA) was added, followed by the addition of 2.07 mg of NHS, and left to react for 15 min at room temperature (RT). Then, 20 mM final concentration of 2-mercaptoethanol (ICN Biomedicals Inc., Costa Mesa, USA) was added to inactivate the EDC. Excess EDC, EDC-byproducts, NHS, and 2-mercaptoethanol were washed out using 30 kDa MWCO Vivaspin® 2 HY Centrifugal Concentrator (Sartorius, Gottingen, Germany) by washing 2 times with 1X phosphate buffered saline (PBS). 0.5 mg/mL of DNA barcode was added to the resulting activated anti-SARS-CoV-2 IgY, mixed well, and then allowed to react for 2 hours at RT. Antibody-DNA was concentrated and purified from free DNA using a 30 kDa MWCO Vivaspin® 2 HY centrifugal concentrator by washing 3 times with 1X PBS. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and ELISA were performed to examine the success of conjugation.

Testing of conjugation efficiency by SDS-PAGE and immunoblot (IB)

SDS-PAGE was performed for the evaluation of IgY-DNA conjugation. 12% acrylamide–bisacrylamide gels under reducing conditions were prepared and samples were loaded as described by Al-Qaoud *et al.* [24]. Electrophoresis was carried out using a running buffer with pH 8.3 at 120 V for 60–120 minutes. The

gel was stained with Coomassie Brilliant Blue R-250 (BDH pool, Dorset, UK), and destained by 20% acetic acid (LOBA Chemie, Mumbai, India) until clear bands were seen. Moreover, 3% agarose was prepared and samples were loaded to identify conjugation based on ethidium bromide staining of nucleic acid. For IB, the SDS-PAGE gel content was plotted to nitrocellulose membrane, and blocked with 3% bovine serum albumin (BSA). Purified chicken IgY was added to the membrane and incubated for 1 hour at RT. After washing the membrane, anti-chicken IgY (mouse origin) conjugated to HRP (MYBioSource, San Diego, USA), diluted 1:1000 in PBS-Tween, was added and incubated for 1 hour at RT. Finally, tetramethylbenzidine (TMB) ready substrate (Sigma Aldrich Inc., St. Louis, USA) was added until color development.

Multiplex IPCR implementation and application on recombinant antigens

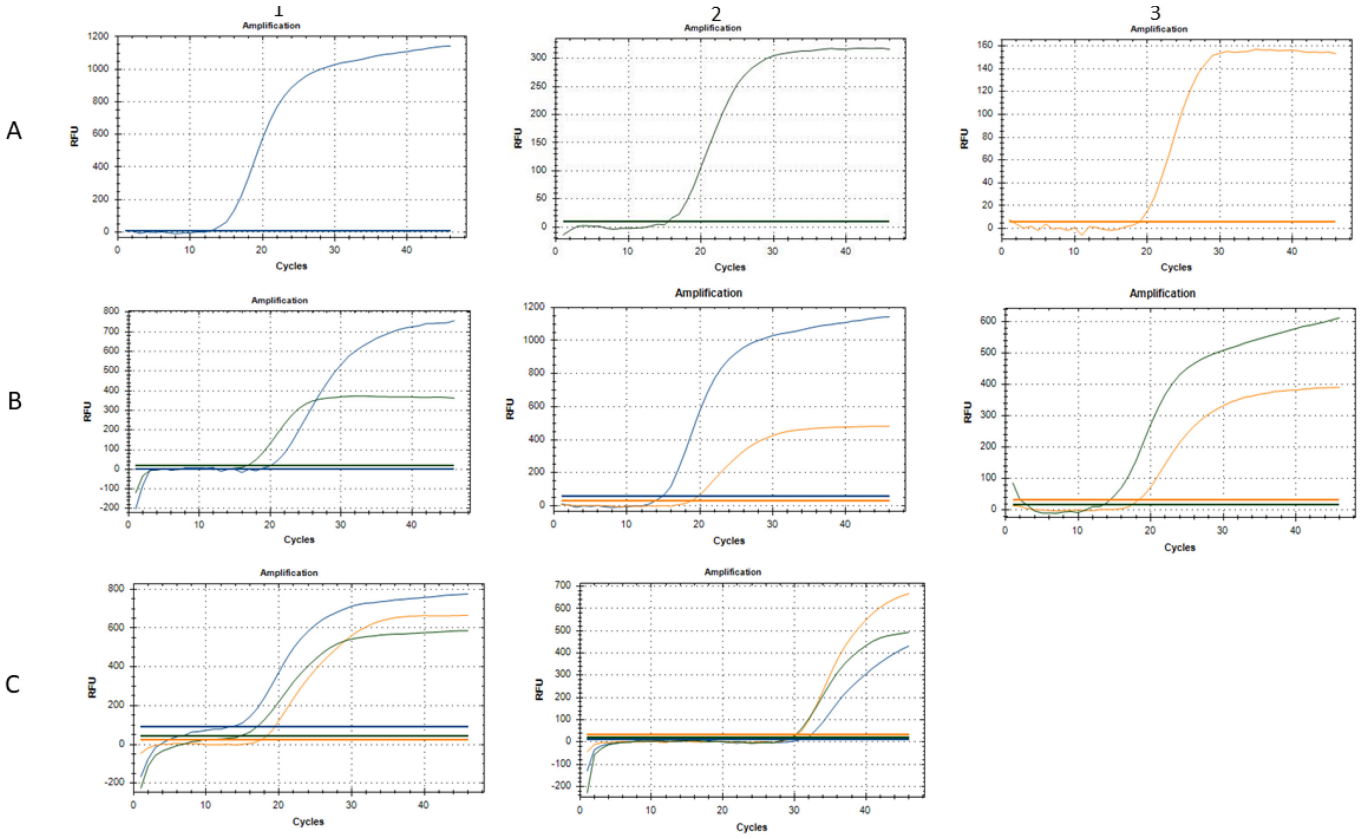
Hard-Shell® 96-Well PCR Plates (Bio-Rad Laboratories, Inc., Hercules, USA) were coated with 10 µg of recombinant N, S, and E antigens in multiplex IPCR format. The plate was incubated at 4 ℃ overnight, then washed 3 times with 0.05% Tween-80 in DEPC-treated 1X PBS. Blocking was done using 3% BSA with incubation at 37 ℃ for 2 hours. The antibody-DNA conjugates were added after a washing step. One type of conjugate was added for uniplex, 2 conjugates for duplex, and a mix of the 3 antibody-DNA conjugates for multiplex IPCR; and the plate was incubated for 1 hour at 37 ℃. The plate was washed thoroughly and carefully after this step to prevent any possible DNA contamination. After that, the RT-PCR program was performed as mentioned previously. IPCR results were considered positive when the setting of any of the tested antigens gave a Ct value below 30 (Figure 1C).

Results

Optimization of the DNA barcodes and primers sets using conventional PCR

PCR amplification of the three DNA barcodes (DNA-1, DNA-2, and DNA-3) showed the expected

Figure 2. Uniplex (A), duplex (B), and multiplex (C) RT-PCR charts displaying Ct value and relative fluorescent unit (RFU) for A-1, DNA-1 (FAM); A-2, DNA-2 (HEX); and A-3, DNA-3 (ROX); B-1, DNA-1 and DNA-2; B-2, DNA-2 and DNA-3; B-3, DNA-1 and DNA-3; C-1, all 3 DNA barcodes' templates plus mix of all primers and probes; C-2, mix of all primers and probes without DNA barcodes (negative control). The amplified DNA barcodes were identified using FAM, HEX, and ROX labeled probes.



Color key: blue (FAM), green (HEX), yellow (ROX). RT-PCR: real-time polymerase chain reaction.

amplicon sizes of 123 bp, 104 bp, and 117 bp, respectively. Furthermore, similar band sizes were revealed when amine-modified primers were used for barcodes amplification (data not shown).

Evaluation of DNA barcodes, primers, and probes by TaqMan RT-PCR

The Ct values of uniplex RT-PCR ranged from 14–20 for the three barcodes (Figure 2A). Moreover, Ct values for duplex and multiplex RT-PCR showed similar Ct range (Figure 2C-1). This indicates that no effect or interferences occurred due to multiplexing. Negative RT-PCR amplification was considered at Ct ≥ 30 (Figure 2C-2). Thus, the PCR for these DNA barcodes demonstrated successful amplification, whether in uniplex, duplex, or multiplex formats (Figure 2).

DNA barcodes-antibodies conjugation

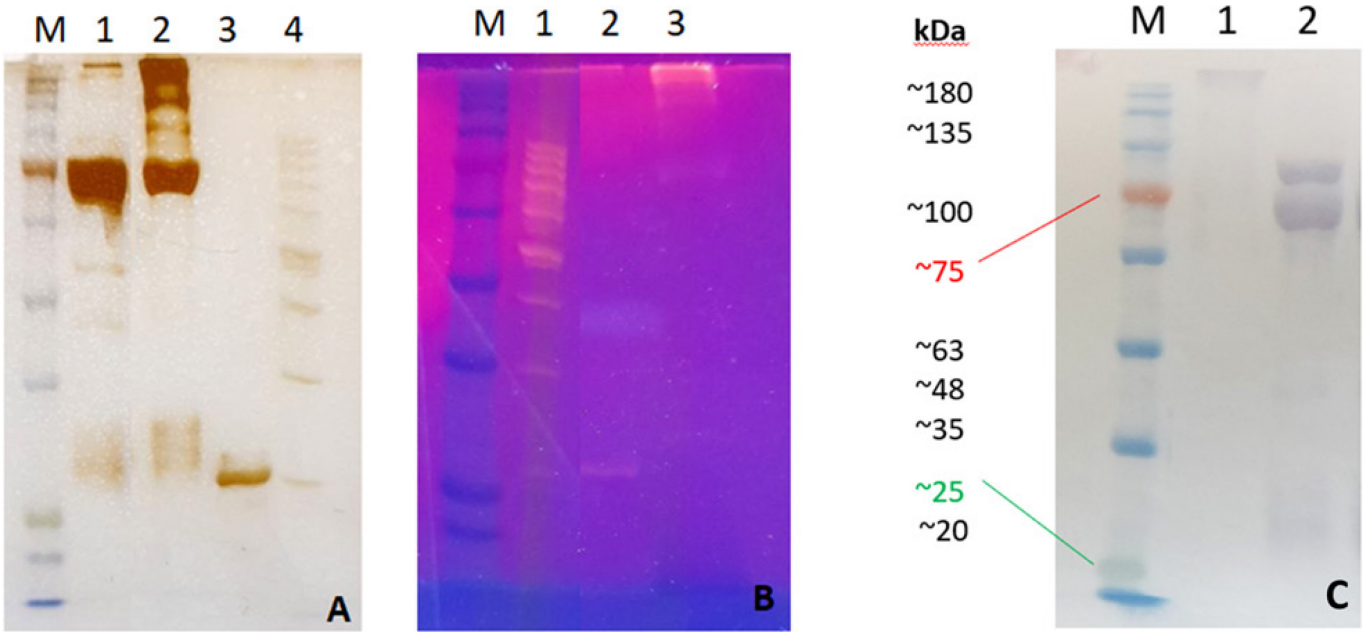
The SDS-PAGEs and Western blot (Figure 3) revealed successful conjugation of IgY with its relevant DNA barcodes. These techniques in addition to the three formats of IPCRs in this study (uniplex, duplex, and multiplex) showed best conjugation yield at 1:1 molar ratio among the antibody and the concerned DNA barcode.

Silver- and ethidium bromide-stained PAGEs (Figures 3A and 3B) verified the IgY-DNA barcodes conjugation. The appearance of a band with higher molecular weight that looks like a ladder pattern (Figure 3A-lane 2, 3B-lane 3, and 3C-lane 1) indicated a successful coupling of IgY with the DNA barcodes (Figure 3). Moreover, ELISA was performed to test the activity of the antibodies following the chemical conjugation processes. The conjugates preserved the antibody's activity in binding to its antigen, as revealed by ELISA (data not shown).

Multiplex IPCR development using commercial SARS-CoV-2 antigens

Commercial N, S, and E recombinant proteins were used to validate the multiplex IPCR for COVID-19 diagnosis. Figure 4 shows the multiplex RT-PCR chart as the final step in multiplex IPCR, indicating the Ct values of N antigen plus Ab-DNA barcode 1, S antigen plus Ab-DNA barcode 2, and E antigen plus Ab-DNA barcode 3; which were 14.45, 18.8, and 15.14, respectively (Figure 4A). However, Ct values of 45.24,

Figure 3. SDS-PAGEs for anti-SARS-CoV-2 IgY before and after conjugation to DNA barcode. **A:** Silver stain displays the protein and DNA profiles of GangNam-StainTM Prestained protein ladder (M), anti-SARS-CoV-2-S IgY (1); anti-SARS-CoV-2 S IgY-DNA barcode conjugate (2); DNA barcode PCR product (3); 100 bp DNA ladder (4). **B:** Ethidium bromide SDS-PAGE with GangNam-StainTM Prestained Protein Ladder (M), DNA ladder (1), DNA barcode PCR product (2), anti-SARS-CoV-2 S IgY-DNA barcode conjugate (3). **C:** Western blot with anti-SARS-CoV-2 S IgY-DNA barcode conjugate (1), and anti-SARS-CoV-2 S IgY (2). Both lanes were incubated with rabbit anti IgY-HRP conjugate.



SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; PCR: polymerase chain reaction; HRP: horseradish peroxidase.

35.65, and 35.2 were observed for the negative controls (Figure 4B).

Discussion

In this study, DNA barcode tagging of chicken IgY against N, S, and E was evaluated to recognize the three antigens in one reaction by developing a multiplex IPCR technique. During the COVID-19 pandemic, obtaining a highly specific and ultra-sensitive diagnostic assay was a big challenge. However, no documented investigations have been conducted to develop IPCR for detecting SARS-CoV-2 [25]. The primary goal of this research was to enhance the specificity of diagnosis. Three antigens of SARS-CoV-2, i.e., N, S and E, were detected by anti-N, -S, and -E chicken IgY that were tagged with DNA barcodes. In addition, the sensitivity was boosted by RT-PCR amplification of the DNA barcodes. It is noteworthy that such a system can be applied for the detection of any analyte if specific antibodies are available.

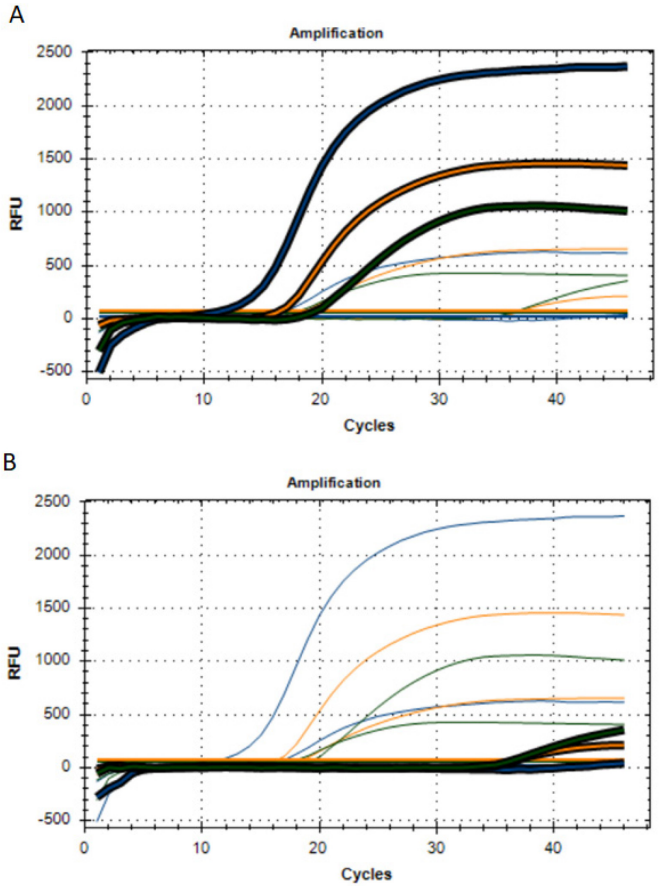
The amazing development in oligonucleotide synthesis has resulted in enhancing diagnostics and therapeutics, enabling the development of biosensors, biological circuits, and other innovations that have contributed to improved human health [26]. Moreover, synthetic DNA barcodes have become a robust tool for species identification [27]. Various studies have utilized DNA barcodes to mark an organism or a molecule [28].

The DNA barcodes in this study were less than 150 bp long, and were generated to tag the previously produced anti-N, -S and -E IgY. These barcodes were well-designed, as demonstrated by their ability to avoid hairpins, self-dimerization, and hetero-dimerization formation during amplification. Hetero-dimerization (intermolecular cross-reactivity) was prevented by prohibiting the presence of complementary nucleotides at both ends of each DNA barcode [29], which also prevented primers and probes from binding to off-target sites, resulting in precise DNA barcode detection and amplification. The three DNA barcodes were examined by the Basic Local Alignment Search Tool (BLAST) of NCBI, and no significant similarity with other gene sequences were noted. Chemically, Gibbs free energy (ΔG) measurements were around -4, -3, and few were close to -10 kcal/mol; which were in alignment with the principles of thermodynamics, and with IDT recommendations [30,31]. The uniplex, duplex, and multiplex RT-PCR charts demonstrate Ct values of synthesized DNA barcodes, as well as their amplification simultaneously in one reaction.

Chicken immunoglobulin of yolk (IgY) has been known for its higher stability and specificity, as well as being quantitatively abundant compared to IgG [32]. This is the first time that chicken IgY has been coupled to unique oligonucleotides using the carbodiimide (EDC/NHS)-based approach. Nevertheless, the bioconjugation process is critical among different categories of macromolecules, i.e. protein and nucleic acid. It requires comprehensive information about the implemented reaction, including the chemical and biological properties of the reactants, pH and its effect on the stability, reaction duration, and the biological activity of the biomolecules [33]. The EDC/NHS conjugation method was selected due to its site-directed conception [24,34].

Chicken IgY possess two carbohydrates sites at its Fc region [35], which are the antibody conjugation sites for the amine-conjugated DNA barcodes. The molar

Figure 4. Multiplex IPCR detection of N, S and E commercial antigens. **A:** RT-PCR chart for the presence of N, S and E antigens in one well. **B:** RT-PCR chart for the system in the absence of antigens.



DNA barcodes conjugated to anti S, N and E IgY antibodies were identified using FAM, HEX, and ROX labeled probes. The IPCR results were considered positive when any of the tested antigens resulted in a Ct value below 30. IPCR: immune-polymerase chain reaction; N: nucleocapsid; S: spike; E: envelope: RT-PCR: real-time polymerase chain reaction.

ratio of antibody to DNA barcode was 1:2, as recommended by Thermo Fisher Scientific Inc. [36]. The conjugation was verified by IB. However, the possibility of free DNA or antibody remaining still existed, which poses a big challenge for IPCR [37], explaining the need for IgY affinity purification. Nevertheless, SDS-PAGE and IB verified the removal of the free molecules of DNA barcodes and antibodies. Moreover, the presence of ladder pattern in SDS-PAGE and IB may indicate the formation of heterogeneous polymers [38].

The validation of the system using clinical samples faced many obstacles during the development and verification of IPCR based on commercial antigens for SARS-CoV-2. One significant challenge was the lack of positive COVID-19 samples, as the pandemic was declared over. Moreover, safety concerns and doubts about the stability of samples kept in refrigerators hindered the validation plans.

Conclusions

IPCR has proved its robustness in recognizing SARS-CoV-2 antigens based on the antibody specificity and the high sensitivity of RT-PCR. The generation of a multiplex system provides the opportunity to detect multiple antigens in one well, something that is not possible with conventional ELISA. The system designed here can be applied for the diagnosis of any pathogen when specific antibodies are available. Furthermore, multiplexing saves both time and materials.

Acknowledgements

This work was funded by the Deanship of Scientific Research at Yarmouk University (Grant No. 75/2022), and by the Scientific Research and Innovation Fund (SRIF) in Jordan.

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Conflict of interests

No conflict of interests is declared.

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