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REVIEW ARTICLE

Understanding Nucleic Acid Amplification Techniques in the Detection of Influenza viruses in Developing Countries

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Abstract:
Introduction: Early detection of emerging influenza virus variant is a key factor in the WHO influenza Global strategies for prevention and control. Rapid, accurate, inexpensive and portable detection systems are needed for influenza virus diagnosis and surveillance. Such a detection system should easily identify all the subtypes of influenza virus. Degenerate primers and probes designed from evolutionally conserved regions for known influenza A viruses present the best way to identify unknown subtypes of influenza A virus by polymerase chain reaction PCR and array techniques. The isothermal reactions, Nucleic Acid Sequencing Based Amplification (NASBA) and Loop-mediated isothermal Amplification (LAMP) possess great potential for influenza A virus detection especially in developing countries. However, multiplex real-time (rT) or quantitative (q) polymerase chain reaction (qPCR) remains a rapid, accurate and time-saving technique used for influenza virus detection.
Aim: This manuscript explained the principles of nucleic acid amplification techniques commonly used in developing countries.
Methods: Literature search was done in NCBI PUBMED, PUBMED Central and Google Scholar using words and phrases including "Influenza-molecular diagnosis, NAAT", Molecular techniques/ methods, PCR, qPCR, NASBA, LAMP, and DNA microarray.
Results: The underlining principles and basic processes involved in the application of nucleic acid amplification techniques for the detection and epidemiological surveillance of influenza virus were identified and grouped under PCR (RT-PCR and qRT-PCR) and Non-PCR (LCR, pyrosequencing, NASBA, LAMP and DNA microarray) amplifications.
Conclusion: It is hoped that by understanding the techniques and basic principles of Nucleic acid amplifications, less expensive, and more convenient protocols for influenza virus detection and surveillance can be developed
Keywords: Influenza, NAAT, Molecular, PCR, qPCR, Viral diagnosis.

All co-authors agreed to have their names listed as authors.

1. INTRODUCTION

Laboratory detection and surveillance of influenza virus dates back to 1930, but in Africa, surveillance activities were not mentioned in literature until later in the century [1]. It is not that there are no viral activities but there is poor surveillance system due to inadequate information on modern tools that can be used despite the fact that molecular techniques have been used in the detection of influenza virus in man and several species of animals for over three decades [2].

Serological assays can provide information on surveillance but in retrospective because both acute and convalescent sera are always required and are not suitable in the identification of novel subtypes due to lack of reagents. Tissue culture can generate a large quantity of influenza virus for characterization but it requires about 3–7 days and it is labour-intensive. Antigen-based assays like rapid influenza diagnostic tests (RIDTs) can be used for rapid detection in about 10 minutes but they are not so sensitive and cannot distinguish between the influenza A subtypes [3].

More sensitive and specific methods are thus urgently required for epidemic and pandemic prevention and controls based on early detection and classical genomic characterization thus providing knowledge about the molecular basis of the virus evolution, transmission, virulence and infection control as well as for prophylaxis, early treatment and resistance mutations monitoring [3].

Hence, the need for molecular techniques that are fast, accurate and sensitive. Applications of PCR-based techniques, other molecular sequencing/ subtyping and microarray methods need to be properly expanded in developing countries as they are now widely employed in developed countries for both diagnosis and surveillance though with continuous evaluation and optimization [4]for quality control in order to prevent bias and missed diagnoses. This write-up therefore reviewed nucleic acid amplification techniques in the detection of influenza virus.

2. METHODS

Literature search was done in NCBI PUBMED, PUBMED Central and Google Scholar using words and phrases including “Influenza- molecular diagnosis, NAAT”, Molecular techniques/ methods, PCR, qPCR, NASBA, LAMP, and DNA microarray.

3. RESULTS

The underlining principles and basic processes involved in the application of nucleic acid amplification techniques for the detection and epidemiological surveillance of influenza virus were identified and grouped under PCR (RT-PCR and qRT-PCR) and Non-PCR (LCR, pyrosequencing, NASBA, LAMP and DNA microarray) amplifications

3.1. Molecular Methods

Molecular assays on viruses are based on the detection of their core nucleic acid (NA) molecules, be it the DNA or RNA. Generally, there are over 30 different types of molecular biological tests. Some are methods for protein detection while others are nucleic acid methods. The major challenge is comparing them (Table 1) and determining which one is more reliable, specific, sensitive, cost-effective and, if possible, suitable for applications in the field, especially in developing countries [5]. All methods of molecular diagnosis can be simply grouped into two categories: Non-Amplification and amplification molecular techniques. This review will focus on nucleic acid amplification techniques.

3.1.1 Sample Collection.

A correct sample for viral study must be collected before the use of any antiviral therapy and at the peak of viral shedding, 2 to 3 days after clinical manifestation [6] and from appropriate site. For influenza study, samples are usually collected from the nose or throat of mammals or from the trachea or cloaca of birds. In humans, upper respiratory tract samples (in descending order of sensitivity) include: nasopharyngeal (NP) aspirate, NP swab, nasal and throat wash or throat swab [7] but in suspected lower respiratory tract infection, abundant viral particles could be detected in specimen like sputum, endotracheal aspirate, and broncho-alveolar lavage fluid [8]. NP sample is usually collected by inserting Dracon swab into the nostril until resistance is met (about 1 to 2 inches in). In children, nasal wash or aspirate is better [3] while other studies have shown that influenza virus may be detected in blood, urine and stool [6, 9, 10].

In animals like pigs, swabs should be inserted approximately to the depth of 1 cm for piglets 0 to 4 weeks-old, 2 cm for nursery pigs from 4 to 7 weeks old, 3 to 4 cm for fattening pigs > 7 weeks of age in accordance with the recommendation of OIE. Throat samples are usually collected by carefully inserting sterile swab stick into the columnar epithelial layer of the throat before being transferred into sterile cryovial containing 1-2mls of transport medium [11].

Typical transport medium include Hanks’ balanced salt solution supplemented with 0.5% bovine serum albumin or 0.1% gelatin and antibiotics [12] (Cheng *et al.*, 2012) or Minimum Essential Medium (MEM) or Dulbecco’s Modified Eagle Medium (DMEM) viral transport medium containing penicillin-streptomycin antibiotic and amphotericin B antifungal agents to prevent desiccation, death, bacterial and fungal growth [13].

3.2 Amplification Techniques

Amplification of nucleic acid is a gold standard technique for analysing a tiny amount of nucleotides in molecular biology, clinical diagnostics, food safety, and environmental testing. It is one of the most valuable techniques in nucleic acid detection. It is a standard method for the detection and sequencing of small quantity of nucleic acid like nuclear DNA, mitochondrial

DNA (mtDNA), cytosolic DNA, messenger RNA (mRNA), ribosomal RNA (rRNA), and a series of non-coding RNAs (ncRNAs) as it can amplify less than 10 target copies, significantly improving assay sensitivity in life science research and molecular diagnostics [14, 15].

RNA amplification is usually performed after the synthesis of complementary DNA (cDNA) from RNA template by reverse transcriptase (RT) enzyme in a process known as reverse transcription [15]. Different commercial assay options are available to detect viral nucleic acid or to amplify and detect thereby becoming the standards for diagnostic virology, supplanting the traditional virus culture and antigen detection techniques [16]. The biochemical mechanisms of most of the techniques are based on target, signal or probe amplification processes [17] and can be classified into PCR and Non-PCR based amplification methods.

3.2.1 PCR-Based Methods

3.2.1.1. Conventional PCR/ Reverse Transcriptase-PCR

Polymerase chain reaction (PCR) in the words of Kary Mullis is a technique that "allows you to pick the piece of DNA you are interested in and to have as much of it as you want" [18]. It is a nucleic acid amplification method that derived its name from the word "polymerase" because the only enzyme used in the reaction is DNA polymerase and called "chain" since the products of the first reaction become substrates of the subsequent reactions [18, 19].

PCR uses short designed complimentary DNA primers flanking the target part (conserved region) of the genome of interest for amplification.The principle is based on nucleotide complementarity and enzymatic exponential synthesis under thermal cycling conditions. PCR-based assay for influenza virus detection was first described in 1991 by Zhang and Evans [20]. It involves conversion of viral RNA into complementary (c) DNA in a process known as reverse transcription using reverse transcriptase enzyme before amplification with specific primers hence the name reverse transcriptase (RT)-PCR.

There are 2 methods of performing RT-PCR, one-step RT-PCR uses oligo-dT or random primers for reverse transcription in a combined reaction with PCR while in two-step RT-PCR, reverse transcription is normally carried out prior to the actual PCR.

All PCR reactions require DNA polymerase that catalysis the reaction by linking each of the nucleotides together to form the amplicons; Nucleotide bases which serve as the building blocks for use by the DNA polymerase; one pair of primer (short complementary DNA fragments) that determines the beginning and end of the target DNA to be detected and amplified. They serve as extension points for the DNA polymerase to build on; DNA template that contains the region of the DNA fragment to be amplified; buffer solution that provides a suitable chemical environment for the DNA-Polymerase; and Mg^{2+} which activates DNA-Polymerase [3, 13].

Amplification processes undergo repeated cycles lasting series of about twenty to fifty cycles in three basic steps that are usually programmed as working conditions in a thermal cycling machine. The mechanisms of the process briefly, include: denaturation, where strands separation occurs by heating up the two complementary strands of the target DNA above their melting temperature at approximately 95°C in order to break the hydrogen bonds that bind one side of the helix to the other [11]. Followed by hybridization or annealing process where the temperature is lowered and the sample is cooled to between 40 to 60°C for specific binding of primers that are complementary to a specific site on each target DNA strand. One primer anneals to a specific site at 3' end of the target strand, while the other primer anneals to a specific site at the 5' end of the other complementary target strand.

Annealing of primers to target DNA sequence (leads to elongation stage where extension of primer-target duplex occurs. It provides the necessary template format that allows heat-stable Taq DNA polymerase at an increased temperature of 72ºC to add nucleotides to 3' end of each primer and produce by extension a sequence complementary (emerging cDNA strands) to the target sequence [21]. It results in doubling of the target DNA with the production of about 10^7 to 10^8 copies after 30 to 40 cycles.

3.2..1.2 Real-Time Reverse Transcriptase-PCR

Real-time (rT) or qPCR is a type of PCR for both the detection and quantification of amplicons. It follows the general basics of conventional PCR but with the measurement of amplicons in real time as the reaction occurs [19]. The fundamental principle in rT-PCR is the ability to monitor amplification with a good detection system and quantify the amplicons as they are synthesized.

There are two methods commonly used to detect and quantify amplicons including: (i) fluorescent (DNA-binding) dyes like SYBR green I that nonspecifically intercalate with double-stranded DNA as it is formed and (ii) sequence-specific DNA probes (e.g. Taqman probe) with fluorescently labeled reporters [19, 21]. The probe consists of oligonucleotides with 5´-reporter dye and 3´-quencher dye. The fluorescent reporter dye e.g. FAM, TET, and VIC is covalently linked to the 5´ end of the oligonucleotide which is normally quenched by TAMRA dye or non-fluorescent quencher linked to the 3´ end [22].

Most real time PCR use dual labeled probe that contain flurophor (reporter) on one end and a quencher molecule on the other. The shape of the un-bound probe allows the flurophor and quencher molecules to sit in close proximity to each other preventing the premature release of fluorescence. The mechanism of the process is that, during PCR reaction, immediately the polymerase acts on a template on which the probe

is bound, the 5'- nuclease activity of the polymerase cleaves the probe. This separates the reporter and quencher dyes, thereby increasing the fluorescence from the reporter (Figure 1). The amount of fluorescence released is measured when bound to the amplicons after each cycle. The quantitative information of the PCR process is obtained after about 30 to 40 cycles by plotting the intensity of the fluorescence signal versus cycle number.

More fluorogenic probes are used simultaneously for identifying multiple amplicons in a single tube in multiplex RT-PCR assay because probes labeled with fluorescent dyes with different excitation and emission spectra allow each target to be measured independently of the others[19]. Real-time RT-PCR (rTRT-PCR) are now widely and increasingly used in influenza A virus detection surveillance because of its high sensitivity, good reproducibility and wide dynamic quantification range with potential for high-throughput screening of a large number of samples. It is a recommended technique by CDC, WHO, and OIE for influenza detection. It is very good for the diagnosis of influenza virus in terms of qualitative and quantitative approach [3].

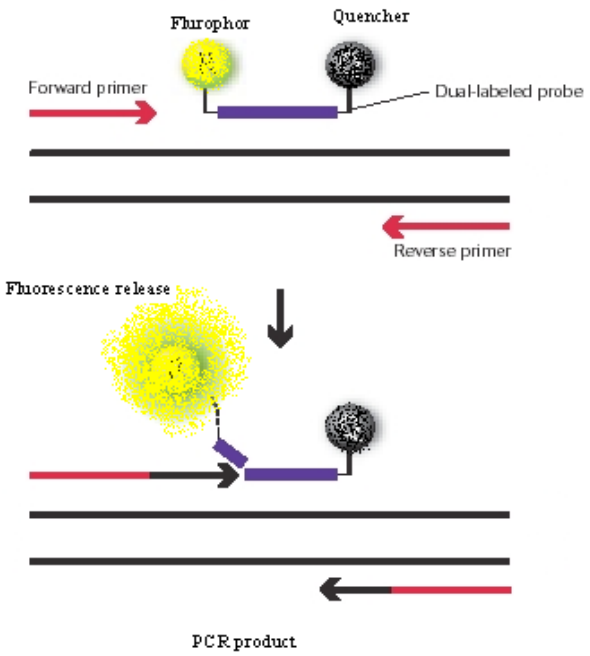

Fig. 1: Principle of Real time PCR using Taqman probe.

(Adapted from [19]).

Several types of PCR have been developed for influenza virus detection including nested PCR that increases the sensitivity of detection by using two sets of amplification primers with one being internal to the other. In duplex, triplex and multiplex PCRs, reactions combine two, three or more sets of primers specific for different targets simultaneously for the detection and discrimination of multiple amplicons in a single tube [23]. Multiplex PCR is a variant PCR. Combined with conventional RT-PCR or real-time PCR, multiplex PCR has been successfully used in the detection, typing and Subtyping of influenza A virus and can provide accurate, sensitive detection for influenza virus surveillance.

3.2.1.3 Pyrosequencing

Pyrosequencing is an on-electrophoretic and non-gel-based amplification method that permits sequencing of smaller fragment of sequences (<100 bp) from amplicons generated by RT-PCR [24-27]. It was developed by Ronaghi *et al* in 1996 [28], based on enzymatic luminometric inorganic pyrophosphate (PPi) detection assay (ELIDA) established by Nyren in 1987 [29].

It operates on a DNA sequencing-by-synthesis principle based on real-time measurement of pyrophosphate released as a result of incorporation of nucleotide into nascent DNA sequence to give direct sequencing data [4, 24].

This relies on the fact that cascade actions of four kinetically balanced enzymatic reactions [(i) Nucleotide incorporation to produce inorganic pyrophosphate (PPi) (DNA polymerase), (ii) Conversion of PPi to ATP (ATP sulfurylase), (iii) ATP use-up to generate light (firefly luciferase) and (iv) degradation of unused molecules (apyrase)] [30] proportionally convert the pyrophosphate to light signals recorded in peaks on graphs called pyrograms[24].

Deng *et al* in 2011 [31] detected and subtyped human influenza A viruses and reassortants with pyrosequencing, describing it as a sensitive and specific procedure that is more rapid and cheaper than using conventional sequencing approaches. It produced a rapid, high-throughput and cost-effective result on a pyrosequencer in the screening of NA inhibitor-resistant influenza A viruses of subtype H1N1, H3N2 and H5N1 [27, 32, 33].

3.3 Non-PCR Amplification-Based Methods

3.3.1 Ligase Chain Reaction

Ligase Chain Reaction (LCR) also known as ligase amplification reaction (LAR) is a probe amplification technique that uses two enzymes: DNA polymerase and DNA ligase to drive its reaction [34]. The principle is based on the ability of DNA ligase to join two complementary probes only when they are specifically hybridized to the target molecules [35, 36].

Ligated probes serve as template for further annealing with each cycle yielding double result of the target nucleic acids [36]. This happens when oligonucleotide probes are annealed to target template in a head to tail fashion with the 3′ end of one probe next to the 5′ end of the second. Follow by joining of adjacent 3′ and 5′ ends by DNA ligase to form a duplicate target strand. Complementary, second primer set then uses the duplicated strand and the original target as templates [37-39]. LCR can be compared to PCR as it performs exponential amplification by polymerization and ligation

of probes as against generation of amplicons by polymerization in PCR. This makes LCR to be more sensitive than PCR, although both methods use thermal cycler and their amplicons can be detected by electrophoresis and real-time processes [40, 41].

3.3.2 Nucleic Acid Sequencing-Based Amplification Method

Nucleic acid sequencing-based amplification (NASBA) also known as self-sustained sequence replication (3SR) is an alternative, rapid and enzyme-based technology that was developed by Compton, J in 1991 from transcription-based amplification system for amplification of nucleic acids[30, 42, 43]. It uses the principle of continuous isothermal (commonly at 41°C) reaction that occurs in two stages: at an initial stage of 65°C (for RNA) denaturation and primer annealing and a cyclic process for target amplification at a constant temperature of 41°C. The entire technique uses a mixture of three enzymes: reverse transcriptase (AMV-RT), ribonuclease-H (RNase H) and RNA polymerase (T7 RNA polymerase) and two target-specific primers: reverse DNA primer P1 containing T7 promoter region and forward DNA primer P2 [44, 45].

Briefly, the initial procedure include: binding of primer P1 to the sample RNA at the 3' end. P1 is extended by the reverse transcriptase enzyme that synthesizes the complementary DNA by RNA/ cDNA hybrid formation. The RNA template is degraded by RNase H (only degrades RNA in RNA-DNA hybrid). P2 then binds to the 5' end of the DNA strand. T7 promoter region is integrated allowing T7 RNA polymerase to bind in order to generate complementary RNA strand. Each synthesized RNA initiates a new round of replication in a cyclic manner thereby yielding an exponential amplification [45-48].

NASBA does not require expensive equipment like a thermocycler, hence, no thermal cycling but uses an ordinary water bath or heating block [17, 45]. It has been shown to be more sensitive than the RT-PCR test in the detection of influenza virus [4] and was used to detect all AIV subtypes (H1–H15) [49]. Also, real-time NASBA has been used to detect influenza A H1N1 and H3N2 and influenza B [50].

3.3.3 Loop-Mediated Isothermal Amplification

Loop-mediated isothermal amplification (LAMP) is a fast and specific one-step amplification method first described by Notomi, Okayama [51]. It is based on the principle of strand displacement reaction under an isothermal condition for the target DNA amplification process [4, 51]. The mechanism consists of three steps: an initial non-cycling, a cyclic amplification and an elongation step [17] using a DNA polymerase with high strand displacement activity; and four different primers including: a forward inner primer (FIP) and backward inner primer (BIP) set and a forward and backward outer primer set that can identify six or more distinct binding sites on the target DNA [30, 45].

Addition of reverse transcriptase makes it possible to amplify cDNA from RNA under the same working conditions [52]. Only a heating block or water bath is required. The detection methods include: manual reading with the naked eye [45], gel electrophoresis and real-time turbidity measurement with turbidimeter [50, 53]. Reverse transcriptase LAMP (RT-LAMP) has been used to detect influenza A and B viruses [54].

Table 1: Comparison of Nucleic acid amplification methods

Technique	Merit	Demerit	Comment
PCR/ RT-PCR	Moderately sensitive and specific; further sequence analysis	Not ideal for high-throughput; quantitative is difficult	Time-consuming for large samples
qPCR	High sensitivity and specificity, quantitative and multiplex detections; high-throughput	Costly probe and equipment; not generally used for further sequencing	Sensitive and reliable for diagnosis and core facilities
Multiplex PCR	Moderately sensitive and specific; Multiple targets per run saving cost and time; downstream sequence analysis	Nonspecific amplification from multiple primers	Suitable for surveillance and subtypes using different segments, and best with qPCR
Pyrosequencing	Accurate and sensitive; high throughout	Time-consuming, limitation of sequencing length	Exceptional for drug resistance markers
NASBA	Moderately sensitive, specific and quantitative	Primer selection and extensive optimization	Cheap instruments, field applications/surveillance
LAMP	Easy, fast and cost-saving	Extensive optimization of primers design for high sensitivity	Regular water bath/ heat block. Applicable in developing countries
Microarray	Sensitive, specific and large amount of targets per run, very high-throughout	Expensive equipment; downstream analysis complex	Fine for surveillance in high-level laboratory or core facility

(ADAPTED FROM [4]).

3.4 Emerging Molecular Systems

3.4.1 DNA Microarray

DNA microarray belongs to the group of emerging diagnostic assay. DNA array also known as DNA chip is a collection of spots attached to a solid support where each spot can contain one or more probes. Microarray is simply the arrangement of biomolecules on a solid surface in order to generate qualitative and quantitative information [55, 56]. It is a technology used to identify

labelled targets by hybridizing them with specific probes and hybridization signals are mapped within the array.

The principle relies on the fact that target molecules like DNA, RNA and proteins can be detected on the basis of complementarity with spotted probes [55]. Hybridization between the immobilized probe and labelled target will generate good fluorescence intensity on a background which can be measured with a fluorescent scanner [55] for data analysis (Figure 2). Briefly, the process involves: attachment of probes to a solid support (glass, nylon, silicon or polypropylene), RNA conversion into cDNA and fluorescent dye labelling using RT-PCR before adding the labelled targets onto known probes for hybridization [5]. Hybridized probes can be detected by a sensitive detection system.

There are different types of microarray techniques differentiated based on the type of probe, solid-surface support and method of detection, including printed double-stranded DNA and oligonucleotide arrays, in situ-synthesized arrays, high-density bead arrays, electronic microarrays, and suspension bead arrays DNA microarray technologies [55, 57].

It has a high throughput because thousands of probes can be spotted on a single slide [56]. It can detect thousands of targets at the same time and can be used to prove phylogenetic relationships between isolates [58]. Influenza viruses are ideal for evaluation by microarrays because of their genetic and host diversity and the availability of an extensive sequence database [59]. It is a technique for identifying and subtyping influenza A virus during surveillance [60].

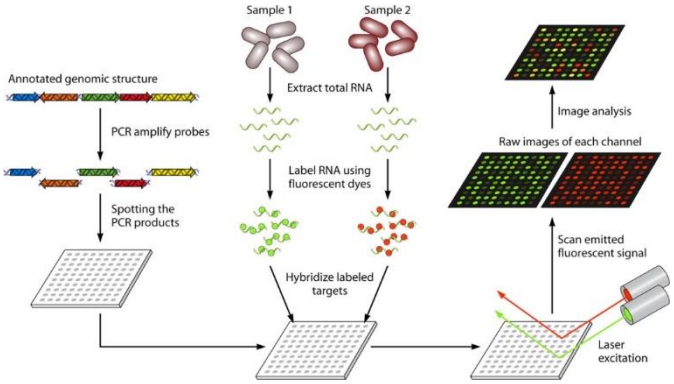

Fig. 2: Workflow summary of printed microarray in 3 parts: Glass slide spotting of probes (PCR amplified or synthesized oligonucleotides); Sample extraction, fluorescent labeling and hybridization; Fluorescent scanning of labeled target nucleic acids hybridized to the probe array.

(Adapted from [61]).

Other emerging molecular systems have generated promising models for the detection of influenza virus including: Proximity ligation assay (PLA), biosensor-based methods, Fo¨rster/ fluorescence resonance energy transfer (FRET)-based methods and nanoparticle-based techniques.

4.0 CONCLUSION

Understanding nucleic acid amplification techniques in the detection of influenza viruses in must begin with proper sample collection for viral study before the use of any antiviral therapy. Highly sensitive PCR and non-PCR based and cost-saving array systems need to be developed for a fast and accurate identification of influenza virus during screening and surveillance. Multiplex and real-time PCR assays can be easily designed for routine screening in most hospital infectious disease centres. Pyrosequencing, combined with other genomic sequencing methods, should be critical focus for screening and subtype identification studies. However, there is need for continuous optimization, standardization and validation of the different nucleic acid amplification techniques before their application from research to clinical diagnosis in developing countries.

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COMPETING INTERESTS

No competing interests exist.

AUTHORS' CONTRIBUTIONS

AAA designed the study, performed the literature search and wrote the first draft of the manuscript; OBS analysed and corrected the manuscript; RKO and BOO assisted in the literature search; AOBO analysed and corrected the manuscript; WAO, and SAO contributed to the study design and supervised the work. All authors read and approved the final manuscript.

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