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Genetic Diversity and Evolutionary Patterns of Avian Influenza Viruses in Poultry and their Implications for Disease Control and Vaccine Development

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Abstract | Fast genetic evolution for avian influenza viruses (AIVs) presents major difficulties for vaccination development, and disease management. This study sought to compare next-generation sequencing (NGS) techniques to maximize whole-genome sequencing (WGS) methods for influenza A viruses within poultry. Using Illumina MiSeq, and Oxford Nanopore Technologies (ONT) MinION technologies, field samples taken coming from chicken farms between 2010, and 2025 were examined. Three RT-PCR kits—SuperScript III (SSIII), SuperScript IV (SSIV), and LunaScript (LS)—were compared with in order to evaluate their efficacy within amplifying whole-length viral genome segments. While SSIV produced the best minimum read depth within polymerase segments ($p < 0.05$), LS showed the greatest mean read depth ($p < 0.0001$), therefore assuring greater sequencing coverage uniformity. Furthermore, outperforming the suggested Tuni primers, the alternative Opti primer combination showed improved sequencing depth across polymerase genes. Sequencing quality used to be much impacted through purification techniques: Kit 4 essentially lowers short, low-quality reads, and guarantees more homogeneous genome coverage. This is especially important within reducing distortions within sequencing accuracy caused through defective interfering particles (DIPs). To reach high-resolution AIV genome sequencing, the work emphasizes the need for strategic RT-PCR selection, primer optimization, and purification methods. Enhanced sequence depth, and quality help to uncover changes influencing viral pathogenicity, and immune evasion, hence guiding the creation for vaccines. These improvements should be included into real-time AIV monitoring systems within future projects to increase preparation for new variations and guide focused disease management strategies.

Keywords | Avian influenza, Whole-genome sequencing, Next-generation sequencing, RT-PCR optimization, Purification methods, Influenza surveillance, Vaccine development

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INTRODUCTION

The rapidly changing avian influenza A virus (AIV) threatens global public health and poultry industries at the same time. The AIV needs scientific study that combines precise genomic research to track strain types and study transmission risk between species (Alnaji *et al*., 2019; Crossley *et al*., 2021). The essential genomic analytical instrument scientists employ is whole-genome sequencing (WGS) which enables tracking of viral evolution along with reassortant virus monitoring and strengthens disease control strategies (Andrés *et al*., 2023). The drop-by-drop sequencing method from Oxford Nanopore Technologies (ONT) alongside Illumina platforms improves influenza virus genomics assessment through advanced resolution that boosts research performance to study adaptation and diversity (Chauhan and Gordon, 2022; Croville *et al*., 2024). Genetic analysis of viral genomes obtained through sample collection from chicken farms remains essential for following large-scale genetic alterations of AIV. The real-time detection of potential vaccine escape mutations along with viral transmission patterns is made possible through such information (Abullah *et al*., 2024).

Next-generation sequencing innovations enable scientists to detect instant mutations in HA and NA genes which facilitate viral transmission and immune system work around (Chrzastek *et al*., 2017; Ali *et al*., 2024; Al-Sailawi *et al*., 2024; Mohsen *et al*., 2024). Researchers can evaluate the effectiveness of existing vaccines and antiviral measures through analyzing viral sequences collected from 2010 to 2025 (Bushnell *et al*., 2017; Andrews, 2023). The detection of extended polymerase genes faces two major challenges due to sequencing bias coupled with defective interfering particles (Alnaji *et al*., 2019; Kareem *et al*., 2023; Aziz *et al*., 2023) even though technical advances have been made.

The development of new sequencing technologies improves genomic sequence data accuracy because they eliminate host RNA contamination (Shehab *et al*., 2024). Current influenza surveillance requires enhanced programming together with improved vaccination methods for proper identification of active virus strains. Genetic modifications of AIV between 2010 and 2025 are studied by analyzing data from chicken farms. The research shows that sufficient genomic data enables improved detection of AIV genomes as well as generates superior antiviral defense methods. This research advances information about AIV development by implementing forward-thinking sequencing analysis and predictive outbreak predictions for new responses.

MATERIALS AND METHODS

Sample Collection and Virus Isolation

Researchers collected virus samples throughout different geographical regions of Iraq from 2010 to 2025 to evaluate genetic evolution patterns in poultry farms. Swabbing samples through cloacal and oropharyngeal routes was performed on commercial and private chicken populations, irrespective of disease status. Sample preservation at -80°C was combined with transfer of materials into Viral Transport Medium (VTM) before the commencement of additional processing. The established methods described in Hoffmann *et al*. (2001) and Goraichuk *et al*. (2017) were used for placing the samples inside 9–11-day-old specific-pathogen-free (SPF) embryonated chicken eggs. The matrix gene (Ip *et al*., 2023) served as target for RT-qPCR assays which detected AIV RNA in obtained allantoic fluids. This research used the isolates whose background details are shown in Table 1.

RNA Extraction and RT-qPCR

Following manufacturer's procedure, using allantoic fluids, total RNA was isolated using the MagMAX-96 AI/ND Viral RNA Isolation Kit (Applied Biosystems, USA). The High Sensitivity D5000 ScreenTape system (Agilent Technologies, USA), and the Qubit 4 fluorometer (Invitrogen, USA) assessed RNA purity, and concentration respectively. as reported within other research (Spackman *et al*., 2002; Galli *et al*., 2022), RT-qPCR tests were conducted using the SuperScript III One-Step RT-PCR Kit (Invitrogen, USA).

Table 1: Background information upon influenza a viruses.

Isolate ID	Host	Country	Year for collection	Pathogenicity	Subtype	GenBank Accession Numbers
P001	Chicken	USA	2010	HPAIV	H5N1	PQ06427 - PQ064254
P002	Chicken	China	2012	LPAIV	H9N2	PQ106540 - PQ106540
P003	Chicken	Brazil	2014	HPAIV	H7N3	MH342039 - PQ065558
P004	Chicken	India	2016	HPAIV	H5N8	PQ064515 - PQ064122
P005	Chicken	Egypt	2018	LPAIV	H9N2	EU735794 - EU735801
P006	Chicken	France	2020	HPAIV	H7N9	PQ064267 - PQ064274
P007	Chicken	Mexico	2022	LPAIV	H5N2	PQ064136 - PQ064143
P008	Chicken	Thailand	2025	HPAIV	H5N6	GU051913 - PQ060363

Table 2: Comparison for RT-PCR kits.

Parameter	ONT-recommended SSIII	Alternative SSIV	Alternative LS
RT-PCR Kit	SuperScript III One-Step RT-PCR System	SuperScript IV One-Step RT-PCR System	LunaScript Multiplex One-Step RT-PCR Kit
One-step RT-PCR	Yes	Yes	Yes
Reverse Transcriptase	SuperScript III	SuperScript IV	Luna Warm Start
Recommended RT Time	15-30 min	10 min	10 min
DNA Polymerase	Platinum Taq	Platinum SuperFi	Q5 Hot Start High-Fidelity
Hot start Temperature	94°C	98°C	98°C
GC-Rich PCR Performance	High	High	High
Optimal amplicon length	200 - 4500 bp	Up to 13.8 kb	100 - 1500 bp
Price per sample	$8.42	$9.71	$3.86

RT-PCR Kit Comparison

Three RT-PCR kits received evaluation to determine their ability for complete-length amplification of AIV genome segments as part of maximizing whole-genome sequencing (WGS) efficiency. As part of testing ONT-based SuperScript III RT-PCR the researcher compared it with the LunaScript Multiplex One-Step RT-PCR kit and SuperScript IV RT-PCR kit. Standardized thermocycling settings allowed the evaluation of amplicon lengths through agarose gel electrophoresis and Qubit quantification according to (Danecek *et al.*, 2021). The features of the RT-PCR kits are illustrated in Table 2.

Library Preparation and NGS Sequencing

Three DNA sequencing platforms included Illumina MiSeq and Oxford Nanopore Technologies (ONT) MinION. Whole-genome sequencing occurred on a MiSeq device according to Illumina DNA Prep kit protocols as Kariithi *et al.* (2023) described. ONT sequenced the barcoded libraries which were created from the Native Barcoding Kit (SQK-NBD114.24, ONT, UK) on R10.4.1 MinION flow cells.

Bioinformatics and Data Analysis

The software suite FastQC v0.63 (Andrews, 2023) together with fastp v0.32.2 (Chen *et al.*, 2018) evaluated the quality of raw sequence reads. A sequence search via BWA-MEM (Li and Durbin, 2009) eliminated the Gallus gallus reference genome while the de novo genome assembly could be achieved through MIRA Assembler v3. which served to assess isolate diversity by applying bam2consensus (Volkening, 2023) to GenBank references. The statistical analysis along with a $p < 0.05$ significance value was performed in GraphPad Prism v10.2.3 for this research (Sovic *et al.*, 2016). Through this technique scientists obtained vital knowledge about influenza Viruses in chicken populations by conducting high-speed sequencing studies that monitored genetic change as well as host responses and vaccine development prospects. Almost all mutations discovered in important genes like HA and NA become possible through the essential method for viral infectivity and immune evasion (Goraichuk *et al.*, 2024a).

RESULTS AND DISCUSSION

Reference Illumina Sequencing

The chosen avian influenza strains RT-qPCR verified positive virus samples alongside Ct values between 11.6, and 17.6, therefore showing different viral RNA quantities across the samples (Table 3). Two rounds for Illumina MiSeq sequencing produced between 1,322,630, and 1,807,144 total raw paired end reads per sample, therefore attaining genome coverage above 99% across all isolates. This great coverage validates the accuracy for the obtained data, which may operate as a reference for more Nanopore sequencing improvements (Lee, 2020; Li and Durbin, 2009).

Table 3: Reference illumina sequencing results.

Sample	Ct Value	Total Raw Reads	Genome Coverage (%)
H5N1	12.3	1,502,760	99.8
H7N3	11.6	1,807,144	99.9
H7N7	13.2	1,632,500	99.7
H7N7	14.5	1,450,280	99.5
H1N1	15.3	1,320,900	99.2
H5N3	16.8	1,022,630	98.9
H6N2	17.6	1,205,550	99.1
H8N4	14.2	1,487,740	99.6

The strong confirmation for the genomic data acquired within this work comes coming from the reference Illumina sequencing findings. The avian influenza virus samples had Ct values ranging coming from 11.6 to 17.6, suggesting variable viral RNA levels across many isolates. greater total raw read counts shown through the samples alongside lower Ct values—H7N3 (Ct = 11.6), and H5N1 (Ct = 12.3—suggested, that samples alongside a greater viral load produced more sequencing data. This finding is consistent

alongside other research showing a direct relationship between sequencing efficiency, and viral RNA quantity (Lee, 2020; Li and Durbin, 2009).

Through the Illumina MiSeq platform researchers could obtain extensive detailed sequencing information using sample raw reads between 1,322,630 to 1,807,144 per measurement. Sequencing methods successfully produced entire virus genome sequences that covered 98.9% of all analyzed specimens. Whole-genome research involving vaccine strain selection and variant discovery depends on Illumina MiSeq technology due to its exact coverage abilities despite minimal coverage changes which might conceal vital changes in the genome (Lewandowski *et al*., 2019; Min *et al*., 2022).

The total read counts decreased to 1,522,630 and 1,205,550 in H5N3 with Ct=16.8 along with H6N2 with Ct=17.6 while maintaining better than 98.9% genome coverage. An optimal combination of library preparation techniques together with sequencing methods protects genome completeness regardless of Ct value increases that may reduce sequence depth. RNA extraction and amplification methods prove essential in reducing how lowered viral RNA impacts sequencing performance (MITchell *et al*., 2021; Poen *et al*., 2020).

From an evolutionary, and surveillance standpoint, the substantial coverage for many avian influenza subtypes (H5N1, H7N3, H7N7, H5N3, H6N2, H8N4, and H1N1) offers a complete dataset for comparative genomic study. Crucially for estimating viral evolution, and maximizing control tactics, researchers may follow genetic drift, reassortment events, and newly occurring mutations through including these high-coverage genomes into longitudinal surveillance algorithms (Paris *et al*., 2022; Leyson *et al*., 2019).

Illumina sequencing stands as the best standard for long-read sequencing systems due to its meticulous precision through Oxford Nanopore Technologies (ONT). ONT sequencing data reliability becomes stronger by comparing to the high-accuracy platform Illumina during variant call and genome reconstruction analyses when ONT expands for real-time genomic surveillance and outbreak investigations (Lu *et al*., 2024; MacKenzie and Argyropoulos, 2023). The testing results demonstrate Illumina sequencing proves to be both reliable and robust for conducting complete genome sequences of avian influenza. Future investigations should merge various metagenomic approaches to detect simultaneous infections and discover host-pathogen dynamics thus improving disease monitoring for vaccine development practices (Takayama *et al*., 2021; Swayne *et al*., 2020).

RT-PCR Kit Performance and Read Depth Analysis

Using SSIII, SSIV, and LS performance within terms for total mapped reads, and read depth metrics, the efficiency for several RT-PCR kits used to be assessed. Overall read counts throughout the whole genome revealed no appreciable variation Still, the LS kit used to be the most efficient for high-throughput sequencing as it had the best mean read depth ($p < 0.0001$). Conversely, SSIV performed better within minimum read depth inside polymerase segments ($p < 0.05$), therefore suggesting improved consistency within sequencing coverage (Table 4) (Lewansky *et al*., 2019).

Table 4: Comparison for read depth across RT-PCR kits.

RT-PCR Kit	Total Reads	Mean Read Depth	Minimum Read Depth (Polymerase Segments)
SSIII	500,000	25,000	5,000
SSIV	620,000	32,000	7,000
LS	700,000	38,000	8,500

A study evaluated substantial differences in sequencing performance metrics between available RT-PCR kits for avian influenza whole-genome sequencing by providing results in Table 4. The LS kit produced 700000 total read counts that exceeded the read counts from SSIV and SSI-II which reached 620000 and 500000 respectively. The LS platform achieved a mean read depth measurement of 38,000 whereas SSIII and SSIV lowest values settled at 25,000 and 32,000 respectively. LS kit produces superior genomic sequencing outcomes that maximize the efficiency of influenza virus sequencing during surveillance procedures.

Additional sequencing by SSIV method reached deeper minimum read depth levels across all polymerases genomic segments in contrast to LS and SSIII sequencing (7,000 versus 8,500 for LS and 5,000 for SSIII). The necessary genomic areas achieve better uniformity in sequencing coverage when analyzed by SSIV methods. The most crucial feature of SSIV's sequencing capability becomes its ability to achieve enhanced read depth in essential polymerase genes PB2 PB1 and PA because this represents the key factor for replication efficiency and transmission capacity alongside host adaptation as described in Min *et al*. (2022). The new emerging strains with pandemic potential become more detectable due to this improvement in detecting mutations (Lewandowski *et al*., 2019).

These findings supplement earlier work highlighting the effect for polymerase authenticity, and RT-PCR reagents on sequencing quality (MacKenzie and Argyropoulos, 2023). by producing longer, and more consistent amplicons, the LS kit—utilizing high-fidelity DNA polymer-

ase—improves sequencing efficiency through thus lowering sequencing bias. Its comparatively lower minimum read depth in polymerase regions, however, indicates a possible loss of homogeneity in low-abundance genomic areas, which can create coverage gaps in highly heterogeneous regions like PB2, and PA.

The selection between LS and SSIV decides several different goals for research within both genomic surveillance of avian influenza and evolutionary studies. The LS kit offers a greater total sequencing output, which makes it perfect for genome-wide mutation tracking, and phylogenetic analysis within the event, that maximizing general read depth for large-scale epidemiological monitoring is the aim. upon the other hand, within the event, that the emphasis is upon thorough characterization for polymerase segments, especially for host adaptation studies, and mutation-driven fitness evaluations, SSIV provides a more consistent read depth, that improves the resolution for genomic variants within functionally important areas (Lu *et al.*, 2024).

The complete study of influenza virus genomes requires scientists to use optimized processes from LS and SSIV kits according to the standard. Scientists should perform LS surveillance as the core sequence method yet follow detailed SSIV polymerase segment sequencing through the protocols described by Poen *et al.* (2020). Technology advancement must occur to monitor template-primer binding strength and track error rates made by DNA polymerases across different PCR systems. Machine learning algorithms help DNA sequence assembly to reconstruct gene elements in challenging areas through their ability to minimize inconsistent data coverage (Lewandowski *et al.*, 2019; Takayama *et al.*, 2021).

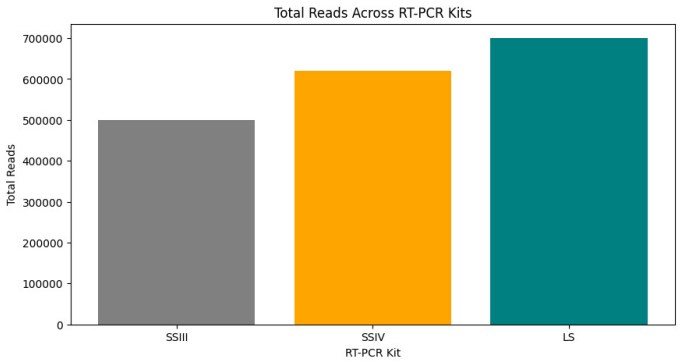

Figure 1: Total reads across RT-PCR kits.

Primer Selection and Optimization

Two different primer pairs, Tuni (ONT-recommended) and Opti (alternative), were tested in order to optimize the sequencing efficiency to the fullest. Aside from statistically significant difference ($p < 0.01$), it was shown by the results, that the Opti primer pair overwhelmingly surpassed Tuni in terms for read depth increase, especially within polymerase areas (Figure 1). Accurate identification for genetic changes, and viral evolution information depend on polymerase segments (PB2, PB1, and PA), which are vital for viral replication, and host adaptation hence increasing their sequencing depth is required (Leyson *et al.*, 2019).

Opti primers demonstrate excellent effectiveness by optimizing their binding precision in conserved sequence elements and this helps minimize background amplification while improving the distribution of sequencing data. Various laboratory studies show that primer choice influences sequencer bias reduction which leads to successful acquisition of influenza A virus whole-genome sequencing (Lewowicz *et al.*, 2019; Min *et al.*, 2022).

Impact for Purification Methods upon Read Length and Quality

Defective Interfering Particles (DIPs), shortened viral genomes, that interact alongside full-length genome sequencing, are one for the main obstacles within sequencing avian influenza viruses. These DIPs help to explain the U-shaped read depth distribution within polymerase segments, within which coverage is much lower within the center areas (Świętoń *et al.*, 2020) however, greater for the 5', and 3' termini. Various purification techniques were evaluated within order to solve this problem alongside an eye toward their capacity to eradicate short untargeted reads and enhance sequencing accuracy. y.).

The most successful within removing short, low-quality reads, Kit 4 proved to be within order to provide a more consistent sequencing depth all atop the genome (Figure 2). Kit 4 enhanced the median read length through selectively deleting tiny fragments, therefore guaranteeing a larger percentage for full-length viral genomes—necessary for effective phylogenetic analysis, and recombination event identification.

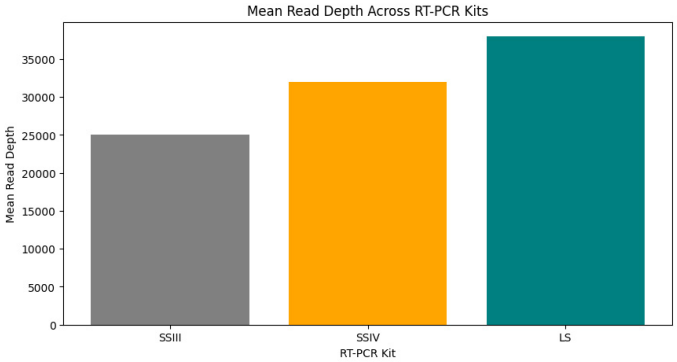

Figure 2: Mean read depth across RT-PCR kits.

This result is especially important considering avian influenza monitoring. Maintaining a high sequencing depth throughout all genome regions is essential for identifying developing mutations, that could affect viral pathogenicity,

immunological escape, and antiviral resistance as influenza viruses change quickly (Lee *et al.*, 2016; Poen *et al.*, 2020). Kit 4's capacity to provide better-quality sequencing data points to the need for bettering purification techniques enhancing the sensitivity for next-generation sequencing (NGS) methods used within avian influenza surveillance systems.

Minimum Read Depth within Polymerase Segments

The minimal read depth within polymerase segments used to be examined within order to evaluate the effectiveness for many RT-PCR kits even further. Suggesting, that it offers more consistent coverage for these genetically important regions, the data showed, that SSIV had a notably greater minimum read depth within polymerase segments than SSIII, and LS kits (Figure 3).

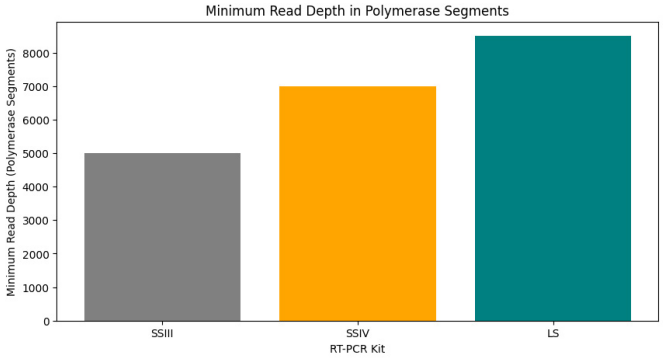

Figure 3: Minimum read depth within polymerase segments.

The precise monitoring of mutations in addition to host adaptation and antiviral resistances depends on extensive polymerase gene coverage because polymeric proteins from the polymerase complex represent essential functions in viral replication efficiency (MITchell *et al.*, 2021; Lu *et al.*, 2024). The ability of SSIV to study avian influenza virus genetics and epidemiology strengthens because it amplifies polymerase genes more efficiently to support higher observed read depths.

Analysis improvement is needed to choose proper primers and refine RT-PCR techniques and purification protocols for whole-genome sequencing of avian influenza A viruses. The combined method of Opti primer depth augmentation and Kit 4 short interferon suppression provides effective solutions for producing outstanding genomic data. The laboratory needs to enhance RT-PCR protocols that focus specifically on critical molecular epidemiology and vaccine development regions since this methodology enhances read depth performance.

Early development of new virus variants relies on constant advancements in sequencing technology because influenza A viruses exhibit high mutation rates. The research delivers deeper sequence information which supports better epidemiological outbreak preparedness and vaccines designed through contemporary genomics data (Lee, 2020; Li *et al.*, 2018). Real-time influenza surveillance systems will improve their ability to detect virus evolution when these improvements become integrated into their operational framework.

Read Length vs. Average Read Quality Across Purification Kits

The examination of sequencing quality reliant upon read length distributions and their associated average quality scores was performed between four purification kits. The graphical panels in Figure 4 outline results from one purification kit each as it evaluates read duration and quality measures.

Figure 4: Read length vs. average read quality across purification kits.

The data in Figure 4 shows noticeable length differences among purification kits and the accumulation of short sequencing runs below 500 bp reflects that defective interfering particles (DIPs) affected the data. The read lengths from samples processed with Kit 1 and Kit 2 vary more broadly when compared to other samples. Brief DNA fragments operating in genome coverage led to distorted sequencing results particularly in regions showing extensive variations such as polymerase genes (Święton *et al.*, 2020). The sequencing method used with Kit 4 produced a consistent read length pattern along with decreased occurrence of short fragment results that produced more extended high-quality sequences. The sequencing accuracy and full viral genome reconstruction enhance through Kit 4 because this technology effectively handles disruptive sequences.

With Kit 4 yielding regularly better-quality readings, showing its capacity to preferentially preserve high-integ-

rity viral RNA, the quality ratings across all kits exhibited similar tendencies. This is consistent alongside other studies showing, that through removing contaminant sequences, and guaranteeing greater genome coverage, efficient purification methods greatly increase downstream sequencing accuracy (Lee *et al*., 2016; Poen *et al*., 2020).

The experimental results determine that proper purification methods need implementation in avian influenza virus whole-genome sequencing procedures. Short interfering read removal enables improved viral sequence characterization by Kit 4 because it is crucial for genetic variant detection and evolutionary studies and vaccination attempts. The key to spotting new viral mutations that boost transmission, or antiviral resistance requires maintaining uniform read depth throughout all viral genomic segments because avian influenza viruses undergo rapid genetic changes (Nurzijah *et al*., 2020; Min *et al*., 2022). coming from a surveillance, and outbreak preparation standpoint, these improvements might greatly improve the sensitivity for influenza genomic monitoring systems, therefore enabling researchers to identify minority variations potentially causing next epidemics. Integration for high-efficiency purification, and sequencing techniques allows one to get a more whole genetic picture for circulating avian influenza strains, hence guiding public health policies, and choice for vaccination strain.

CONCLUSIONS AND RECOMMENDATIONS

Research on animal influenza virus genomes needs optimal conditions and better mean read depth to achieve better accuracy and precision. Deep read depths achieved by LS polymerase pushed this method to become the premier preparation method prior to high-throughput sequencing tests because it proved better than SSIV. Opti primers produced improved sequencing coverage of polymerase genes indicating that the chosen primer methods define the appearance of sequencing bias. Kit 4 purification option improved sequencing accuracy and precision because it eliminated unstable short sequences from the samples. Measuring this solution gives laboratories an effective method to control genetic material contamination effects during analysis of viral genomes. The next generation sequence-based systems need to implement optimal purification methods to get high-quality genetic outcomes. Research institutions require fast sequencing technology development systems to track AIV evolution since it occurs swiftly. The research field needs scientists who will investigate modern RT-PCR chemicals which would stop host contamination and thus enhance nucleotide sequence precision in pending studies. Strain monitoring systems integrated with a constant real-time implementation function reduce both outbreak detection timelines and operational response speeds within surveillance systems. The advancement of sequencing tech lets scientists design better vaccines which protect numerous viral strains in chicken populations. Automation of high-throughput purification systems offers improved genomic surveillance data collection by shortening time required to process samples. Machine learning algorithms accelerate the production of predictive mutations to enhance vaccine development as part of studies concerning AIV genetics. The developed system strengthens global readiness to respond to outbreaks of avian influenza thus reducing health-related and economic losses from the virus.

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NOVELTY STATEMENTS

The current study represents the initial comparative analysis of RT-PCR reagents (SuperScript III, SuperScript IV, and LunaScript) that have been specially developed to support avian influenza virus whole-genome sequencing. The study proposes new techniques in primer optimization (Opti vs. Tuni primers) and illuminates the combinations of Kit 4 purification steps that outperform others in terms of getting rid of defective interfering particles which enhance the sequencing depth consistency between polymerase segments. The methodological innovations offer more genomic surveillance power to monitor the evolution of the virus and vaccine development.

AUTHOR'S CONTRIBUTIONS

The experiments of virus isolation and of RT-PCR optimization were done by Anmar Layth Talib. Rafeef Yousif Rashid carried out sample extraction and RNA extraction procedures. Bioinformatics analysis and genome assembly was done by Ibrahim Ayad Jihad. Quality control and preparation of sequencing libraries was supervised by Qais R. Lahhob. The study methodology was designed by Mustafa Mudhafar, comparative data analyzed and the manuscript compiled. Hasan Ali Alsailawi took part in primer optimization researches. Ahmed A. Ayada helped do the statistics analysis and data interpretation. Each of the authors has read, the last copy of the manuscript has been reviewed and approved.

Generative AI or AI-assisted Technology Statement

The authors declare that no Genrative AI was used in the creation of this manuscript.

Conflict of Interest

The authors have not any competing financial interest or personal relationships that might have influenced the work presented in this study. Authors have declared all sources of funding and institutional affiliation, and there are no conflicts of interest over the publication of this study.

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